Protocol development for analysis of the *DMPK* repeat in preimplantation genetic diagnosis and the investigation of gene expression in human oocytes and blastocysts

by

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'I, Georgia Kakourou, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.'

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To my parents

Abstract

Myotonic dystrophy type 1 (DM1) is an autosomal dominant disorder caused by expansion of an unstable CTG repeat within the 3'UTR of the *DMPK* gene, which expands further in length during transmission from generation to generation. Prenatal diagnosis is available, although the decision for pregnancy termination can be difficult due to the variable phenotypic expression of DM1. *In vitro* fertilisation with preimplantation genetic diagnosis (PGD), offer another reproductive option for affected couples, which involves genetic analysis and selection of an unaffected embryo to establish a pregnancy. These technologies have also provided access to human gametes and preimplantation embryos and encouraged research aimed at understanding the molecular pathways controlling human preimplantation development.

The first part of this study focused on the improvement of existing techniques for PGD and the development of universal multiplex fluorescent PCR PGD protocols for the efficient and accurate diagnosis of DM1. The second part of the study involved follow-up analysis of DM1 affected and unaffected embryos donated for research with the aim to investigate transmission of the CTG repeat from the affected and unaffected parent to the preimplantation embryo. The final objective was to obtain a global gene expression profile by microarray analysis of human oocytes and blastocysts, with a focus on important functional pathways.

The protocols developed achieved high efficiency and accuracy of diagnosis, reduced the genetic work-up time, overall supporting PGD for DM1 as an effective and practical alternative to prenatal diagnosis. This study also adds to current evidence regarding CTG repeat transmission and provides information on repeat expansion and embryo quality in DM1. A comparison of expression in the healthy oocyte and blastocyst is presented, including the identification of oocyte-unique and blastocyst-unique genes. The microarray data from this study will guide experiments to identify cases where normal gene expression is disrupted.

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2. Materials and Methods

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Abbreviations

AC	Amniocentesis
aCGH	array CGH
ACTB	Beta actin
ACU	Assisted Conception Unit
ADO	Allele dropout
ALB	Alkaline lysis buffer
AMA	Advanced maternal age
ARMS	Amplification refractory mutation system
BLAST	Basic Local Alignment Search Tool
BMP-15	Bone morphogenic protein- 15
bp	base pairs
BSA	Bovine serum albumin
CCND2	cyclin D2
CDM1	Congenital myotonic dystrophy type I
cDNA	Complementary DNA
CGH	Comparative genomic hybridization
CLCN1	Muscle chloride channel
cRNA	Complementary RNA
CTCF	CTC-binding factor
CTNND1	Catenin delta 1
c-TNT	Cardiac troponin T
CUGBP1	CUG binding protein 1
CVS	Chorionic villus sampling
CXCR4	Chemokine receptor 4
dATP	Deoxyadenosine triphosphate
DB	Dissociation buffer
dCTP	Deoxycytidine triphosphate
dGTP	Deoxyguanosine triphosphate
DIG	Digoxigenin
DM1	Myotonic Dystrophy type I
DM2	Myotonic dystrophy type II
DM3	Myotonic dystrophy type III
DMPK	Dystrophia myotonica-protein kinase
DMSO	Dimethyl sulfoxide
DMWD	Dystrophia myotonica-containing WD repeat motif
DNA	Deoxyribonucleic Acid
DNMT	DNA methyltransferase
dNTP	Deoxyribonucleotide triphosphate
DOP-PCR	Degenerate oligonucleotide primed-PCR
DRPLA	Dentatorubral-pallidoluysian atrophy
DTT	DL-Dithiothreitol
dTTP	Deoxythymidine triphosphate
EDTA	Ethylene-diamine-tetraacetic acid
EGA	Embryonic genome activation
ELT	Expand long template
ESHRE	European Society for Human Reproduction and Embryology
EST	Expressed sequence tags
ET	Embryo transfer
FAP	Familial adenomatous polyposis
FEN1	Flap-endonuclease 1
FISH	Fluorescent *in situ* hybridization
FN	False negative
FP	False positive
F-PCR	Fluorescent PCR
FRAXA	Fragile X syndrome
FRDA	Friedreich's ataxia
FSHD	Facioscapulohumeral muscular dystrophy
GC	Granulosa cell
GDF-9	Growth differentiation factor-9
GPX3	Glutathione peroxidase 3
GREM1	Gremlin 1
GV	Germinal vesicle
GVBD	Germinal vesicle breakdown
HA	Heteroduplex analysis
HAS2	Hyaluronic acid synthase 2
hCG	Human chorionic gonadotropin
HD	Huntington's disease
hESC	Human embryonic stem cell
HFEA	Human Fertilisation and Embryology Authority
HKG	Housekeeping gene
HLA	Human leukocyte antigen
ICM	Inner cell mass
ICSI	Intracytoplasmic sperm injection
IR	Insulin receptor
IVF	*In vitro* fertilisation
IVT	*In vitro* transcription

kb	kilobase
LA-PCR	Linker-adaptor PCR
LATE-PCR	Linear after the exponential PCR
LDL	Low density lipoprotein
LH	Luteinizing hormone
MBNL1	Muscleblind-like 1 protein
MDA	Multiple displacement amplification
MI	Meiosis I
MII	Meiosis II
MJD	Machado-Joseph disease
ml	millilitre
mRNA	messenger RNA
MTMR	Myotubularin related protein 1
mTP-PCR	Multiplex TP-PCR
OHSS	Ovarian hyperstimulation syndrome
OMIM	Online Mendelian Inheritance in Man
PB	Polar body
PBS	Phosphate buffered saline
PCNA	Proliferating cell nuclear antigen
PCR	Polymerase chain reaction
PDGF	Platelet derived growth factor
PEP	Primer extension preamplification
PGD	Preimplantation genetic diagnosis
PGH	Preimplantation genetic haplotyping
PGS	Preimplantation genetic screening
PK	Proteinase K
PN	pronucleus
PND	Prenatal diagnosis
PROMM	Proximal myotonic myopathy
PTX3	Pentraxin 3
PVA	Polyvinyl alcohol
RFLP	Restriction fragment length polymorphism
RFU	Relative fluorescence units
RIN	RNA integrity number
RNA	Ribonucleic acid
Rnase	Ribonuclease
RT	Room temperature
SAGE	Serial analysis of gene expression
SBMA	Spinobulbar muscular atrophy
SCA	Spinocerebellar ataxia
SDS	Sodium dodecyl sulphate
SIX5	Sine oculis homeobox 5
SNP	Single nucleotide polymorphism
SP-1	Specificity protein-1
SSCP	Single stranded conformational polymorphism
STR	Short tandem repeat
SUMO	Small ubiquitin related modifier
T-PCR	Tagged random primers PCR
TE	Trophectoderm
TF	Transcription factor
TG	Triglycerides
TGF-β	Transforming growth factor β
TLAD	T7-based linear amplification of DNA
Tm	Temperature of the primer melting point
TOP	Termination of pregnancy
TP-PCR	Triplet repeat-primed PCR
TRD	Transmission ratio distortion
UTP	Uridine-5'-triphosphate
UV	Ultraviolet
WC	Watson-Crick
WGA	Whole genome amplification
ZNF9	Zinc finger protein 9
ZP	Zona pellucida

1. Introduction

1.1 Preimplantation genetic diagnosis

Preimplantation genetic diagnosis (PGD) involves testing of the preimplantation stage embryo, created with *in vitro* fertilisation (IVF) technology, for inheritable chromosomal abnormalities or single-gene defects and transfer of unaffected embryos to the womb to establish a pregnancy. PGD requires a multidisciplinary approach. Couples receive an initial counselling by a clinical geneticist and disease specialists. Following that, doctors and embryologists of the IVF clinic are responsible for counselling on the IVF aspects and performing a successful IVF treatment, while the genetics team are responsible for counselling on PGD and developing and applying a reliable and efficient diagnostic protocol for the embryo stage. PGD is not an easy reproductive option and several difficulties may be encountered during the whole procedure. These include the considerable cost per cycle, the chance of misdiagnosis and the low success rate. In addition, commencing treatment does not guarantee the availability of embryos for transfer. This can be the result of poor ovarian response, failed fertilisation, poor quality embryos, diagnosis of all embryos as affected or having inconclusive results (Sermon et al., 2004). Despite these potential difficulties, PGD is a very appealing option to many patients, particularly those with moral or religious objections to termination of pregnancy (TOP) and those having undergone several TOPs. In fact, in one of the early studies reporting on the patient's view on PGD, 86% of women stated that avoidance of TOP was the main advantage they saw in PGD (Pergament, 1991). This was confirmed in a later study reporting the views of patients who had actually experienced PGD (Lavery et al., 2002). Another group of patients who may choose to undergo PGD are those with infertility problems who therefore have an independent indication for IVF treatment and also carry a genetic disorder (Delhanty et al., 1994;Delhanty and Wells, 2002).

The first established pregnancies following PGD were reported in 1990 for two couples with X-linked recessive disorders, adrenoleukodystrophy and X-linked mental retardation. In these cases diagnosis involved sexing of the foetus by amplification of a Y-specific DNA sequence and selection of female embryos for transfer (Handyside et al., 1990). The first clinical application of PGD for monogenic disorders was for cystic fibrosis in 1992 (Handyside et al., 1992). It is estimated that over 7000 PGD cycles have been carried out to date. During the last eighteen years the practice of PGD has changed

considerably, not only from the technical point of view but also regarding its general application, often being the centre of ethical debates (Kuliev and Verlinsky, 2005;Kuliev and Verlinsky, 2008). PGD is no longer limited to conditions that present at birth, but has also been applied for the diagnosis of late-onset disorders with genetic predispositions, as a screening test for various high-risk population groups (preimplantation genetic screening, PGS) or even for non-disease associated human leukocyte antigen (HLA) testing in order to obtain compatible offspring to affected siblings who require transplantation therapy (Fiorentino et al., 2004;Rechitsky et al., 2004;Delhanty, 2006;Mantzouratou et al., 2007; Renwick et al., 2007). Legislation for PGD practice differs between countries (Soini, 2007). In the UK PGD is strictly regulated by the Human Fertilisation and Embryology Authority (HFEA) and, internationally, PGD data are collected by the European Society for Human Reproduction and Embryology (ESHRE). Over 2000 children have been born worldwide and to date studies on the health of PGD and PGS children have not indicated the PGD procedure to be unsafe (Banerjee et al., 2008;Goossens et al., 2008b;Nekkebroeck et al., 2008).

1.1.1 Embryo biopsy and diagnosis

The first step of the PGD procedure involves puncture or removal of part of the zona pellucida, the thick membrane that surrounds the oocyte or embryo, in order to obtain the cells for genetic analysis (biopsy procedure). Biopsy can be performed using acid Tyrode's solution, laser or mechanical means, at different stages of development, either around the time of fertilisation of the woman's oocyte with her partner's sperm, to allow testing of the polar bodies, or during the cleavage or blastocyst stages of preimplantation embryo development, to obtain blastomeres or trophectoderm cells respectively (Boada et al., 1998;Inzunza et al., 1998;Harper and Thornhill, 2001) (figure 1.1).

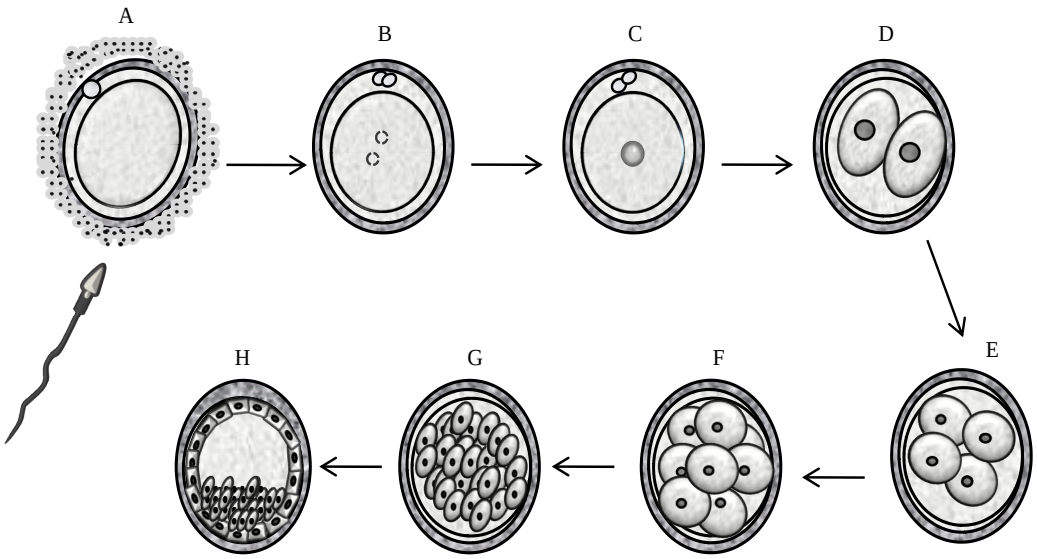

Figure 1. 1: Sequence of events following human oocyte fertilisation. A: Mature oocyte (MII) with first polar body present, surrounded by cumulus cells. B: Fertilisation: formation of the male and female pronuclei (2PN) and extrusion of the second polar body. C: Human zygote. D: First mitotic division. E-F: Mitotic divisions leading to a 4-cell and an 8-cell embryo. G: Morula stage of preimplantation development. H: Blastocyst stage embryo where cells have differentiated into the trophectoderm (the surrounding cells) and inner cell mass.

Polar body (PB) biopsy is the only option available in countries where diagnosis at the embryo stage is not legal; an example of this is Germany, though it is interesting to note that the German public opinion differs to the government regulation (Borkenhagen et al., 2007). PB biopsy is labour-intensive as it requires removal and testing of the first polar body, produced after completion of the first meiotic division, while confirmation of findings is possible by subsequent removal of the second polar body, produced after fertilisation (Sermon et al., 2004). In the case of a maternal defect or an X-linked disorder, PB biopsy permits detection of the mutation-free oocytes; embryos derived from these do not require further testing. With PB biopsy, however, paternally-inherited abnormalities or other defects originating following fertilisation cannot be detected, while, for single-gene disorders PB diagnosis may be associated with problems arising due to meiotic cross-over (Swanson et al., 2007). Additionally, for X-linked or recessive conditions, some of the oocytes that are discarded as “affected”, could lead to unaffected embryos depending on the paternal genetic contribution (Soini et al., 2006).

Cleavage stage embryo biopsy is the most common method used in PGD. It involves blastomere removal on day 3 of development, when the embryo is at the 6-8 cell stage. This is generally considered as the best approach, as the cells at this stage are regarded as totipotent, compaction has not yet occurred and there is sufficient time for the diagnostic protocol to be carried out prior to embryo transfer on day 4 or day 5 (De Vos and Van Steirteghem, 2001). The main debate regarding this method of obtaining cells has been directed on whether one or two blastomeres should be biopsied. Investigators have assessed the impact of one or two-cell biopsy on the diagnosis rate, the risk of misdiagnosis, embryo development and implantation potential. At the present time, as there are not any specific practice guidelines on this issue, each PGD centre follows its own guiding principles when performing cleavage stage biopsy (Lewis et al., 2001;Goossens et al., 2008a;Dreesen et al., 2008).

Finally, blastocyst stage biopsy may be performed on day 5, by removing several cells from the trophectoderm layer, which gives rise to the placental membranes. The availability of more than one cell facilitates diagnosis and increases the overall diagnosis rate, though the occurrence of mosaicism could have consequences on PGD accuracy, particularly for the diagnosis of chromosomal disorders (Kokkali et al., 2007). Higher implantation rates are generally achieved with transfer of human blastocysts rather than transfer of day 3 embryos (Gardner et al., 1998a;Gardner et al., 1998b;Blake et al., 2007). Transfer of blastocysts following blastocyst biopsy has also been associated with an improved implantation rate compared to transfer of blastocysts where cleavage stage biopsy was performed, although notably only 40-50% of embryos develop to the blastocyst stage by day 5 (Kokkali et al., 2007;McArthur et al., 2008). Additionally, with blastocyst stage biopsy, embryo cryopreservation may be necessary to allow time for the diagnosis to be completed (Wilton et al., 2001; McArthur et al., 2005;Magli et al., 2006).

Following embryo biopsy, the polymerase chain reaction (PCR) is commonly used for the diagnosis of single gene disorders. This is discussed in more detail below. The main method for detection of chromosomal abnormalities has been the fluorescence *in situ* hybridization (FISH) technique, performed on human blastomeres since 1992 (Griffin et al., 1992). This technique requires fixing the single blastomere on a slide prior to

labelling with chromosome-specific fluorescent probes. FISH allows detection of abnormalities that may arise in embryos of carriers of Robertsonian or reciprocal translocations as well as other chromosomal rearrangements such as inversions, insertions, deletions, or the formation of ring chromosomes. The technique has also been employed as a screening test to detect numerical chromosomal abnormalities in embryos from couples of advanced maternal age (AMA), or couples with repeated failed IVF cycles (three or more) or repeated miscarriages, as the above have been associated with chromosomal aneuploidy. This test is known as preimplantation genetic screening (PGS) (Fragouli, 2007). The main drawback with FISH has been the restricted number of probes that can be used in the same experiment (up to 5). Several PGS protocols have been reported, generally allowing for testing of up to 15 different chromosomes following consecutive FISH rounds on a single blastomere (Baart et al., 2007a;Baart et al., 2007b;Mantzouratou et al., 2007). The anticipated positive clinical benefit of the PGS treatment has been under debate (Cohen et al., 2007). To overcome the limitations of FISH, a technique known as comparative genomic hybridization (CGH), which allows the simultaneous analysis of all chromosomes in a cell, has been optimised to work on single cells (Wells et al., 1999). The technique has been applied to blastomeres from day 3 embryos, as well as to polar bodies and blastocyst stage embryos (Wells and Delhanty, 2000; Wilton et al., 2001;Wells et al., 2002; Fragouli et al., 2006a;Fragouli et al., 2006b;Fragouli et al., 2008). Its main limitations have been the long time required to obtain a result (4-5 days) and the need for particular expertise to accurately carry out the analysis. To allow enough time for completion of the analysis, embryos are cryopreserved and, following diagnosis, the selected embryos are thawed for transfer in a new treatment cycle. This methodology has been associated with reduced implantation of thawed embryos, but the constant improvement of cryopreservation techniques is gradually overcoming this drawback (Sher et al., 2009).

Another more recent development, array CGH (aCGH), overcomes the above limitations as the results can be obtained within 24 hours and the analysis can be easily automated (Hu et al., 2004;Le et al., 2006). aCGH, using oligonucleotide probes, has already been applied clinically (Hellani et al., 2008). Other microarray platforms have also become available for the detection of aneuploidy, although further work is needed to optimise their use and overcome problems associated with variation in performance, resolution, diagnostic accuracy, interpretation of results and high cost, to encourage their wide clinical application (reviewed in Wells et al., 2008). It is, however,

noteworthy that the single nucleotide polymorphism (SNP) arrays present an opportunity for simultaneous detection of chromosomal and single gene disorders, which has traditionally been a challenge for PGD.

1.1.2 PGD for single gene disorders

Following embryo biopsy the PGD procedure involves gentle lysis of the membranes of the biopsied cells in order to make the DNA accessible. Since the advent of PGD, various protocols have been tested for their efficiency in single cell lysis. Methods of lysis prior to performing PCR for the diagnosis of single gene disorders have included consecutive rounds of freezing and thawing in distilled water, boiling, the use of alkaline lysis buffers (ALB), or the use of buffers containing proteinase K (PK) and detergents. The ALB and PK lysis methods are currently the most widely used (Thornhill et al., 2001). Following cell lysis, PCR is used for amplification of the DNA, around 6pg from a single cell, to a level where mutation detection techniques can be applied. Through the years, the single-cell PCR strategies have evolved with the purpose of achieving better diagnostic efficiency, more accurate diagnostic results and, consequently, a lower misdiagnosis rate.

The main aim has been to minimise the PCR problems that are exacerbated when amplifying from a single genome, as opposed to highly-concentrated DNA samples used in routine PCR. These include **amplification failure**, **allele dropout** or **contamination** and can result either in failure to make a diagnosis or in misdiagnosis. Bearing in mind that single-cell PCR amplification can be performed only once, taking measures for prevention of the above becomes critical, especially as several misdiagnoses have been reported since the introduction of PGD (Goossens et al., 2008a).

1.1.2.1 Single-cell PCR features

1.1.2.1.1 Amplification failure

The most common causes for amplification failure include loss of the cell during transfer to the PCR tube, biopsy of an anucleate or poor quality (lysing or degenerating) cell, inadequate cell lysis or use of a suboptimal PCR protocol. Amplification failure reduces the numbers of diagnosed embryos and therefore, the chances of embryo transfer, implantation and pregnancy. Care should be taken during protocol design for PGD, so that lack of amplification does not mimic an 'unaffected' result, in order to avoid misdiagnosis. Standard PGD practice should, therefore, entail careful monitoring of the cell status during the biopsy procedure, as well as cautious single-cell manipulation and use of optimised and well tested single-cell protocols. In most studies, the amplification success rate for single buccal cells or single lymphocytes is around 90-95% and, similarly, amplification failure has been estimated to occur in approximately 10% of isolated blastomeres (Harper and Wells, 1999).

1.1.2.1.2 Allele dropout (ADO)

ADO is a phenomenon generally affecting 5-20% of single cell amplifications, whereby one of the two alleles in a heterozygote sample fails to amplify to a detectable level. The cause of ADO is mainly technical, due to inefficient priming during PCR, and has also been associated with poor embryo quality. ADO can affect either of the alleles in a heterozygous cell. When it occurs on amplification of the mutated region, it can be responsible for false negative and false positive diagnostic results. ADO of the mutant allele during PGD for a dominant disorder can lead to transfer of an affected embryo. In PGD for recessive disorders, ADO of the unaffected allele can reduce the number of heterozygous (carrier) embryos detected (figure 1.2). For recessive disorders that are due to a combination of two different mutations (compound heterozygotes), an embryo might be given a carrier rather than affected diagnosis in case of allele dropout at one of the loci (Wells and Sherlock, 1998).

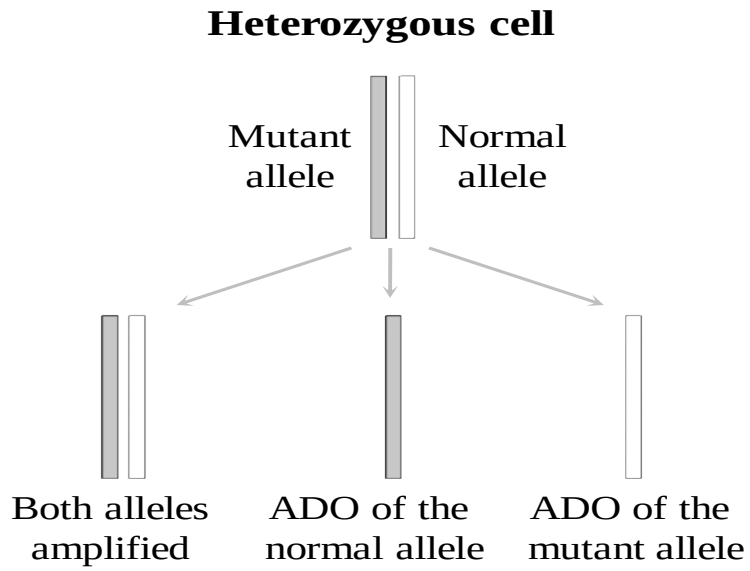

Figure 1. 2: Allele Dropout (ADO). Failure of amplification of the normal allele mistakenly leads to erroneous interpretation as a homozygous affected genotype. When the mutant allele fails to amplify the genotype appears to be that of a homozygous unaffected individual.

Various techniques aimed at reducing the chance of ADO have been applied in PGD, for example increasing the PCR denaturation temperature for the first ten amplification cycles or using alternative cell lysis buffers (Ray and Handyside, 1996;el Hashemite and Delhanty, 1997;Piyamongkol et al., 2003). In addition, the highly sensitive technique of fluorescent PCR (F-PCR), where primers in a PCR reaction are end-labelled with fluorescent molecules and products are analyzed on a fluorescent capillary detector, has been used to detect cases of preferential allele amplification, which, with conventional PCR, would appear as ADO (Findlay et al., 1995). Finally, the use of multiplex PCR allows the simultaneous amplification of a polymorphic intragenic or linked marker along with the mutated region. The marker's phase allele, the allele inherited on the same chromosome with the mutation, is detected by linkage analysis and its presence confirms the presence of the mutation (Rechitsky et al., 1998;Sherlock et al., 1998). It is important that linked polymorphic markers are in close proximity to the gene to ensure that the chance of the mutation and the phase allele having separated by meiotic recombination is reduced. The higher the number of linked polymorphic markers available for the gene under study, the higher the chance that, should ADO of the mutant allele occur, it will be detected (figure 1.3).

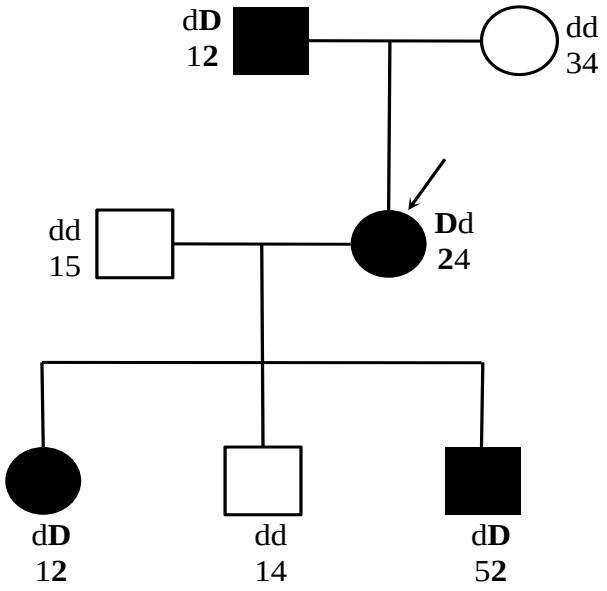

Figure 1. 3: Detection of the phase allele in a two-generation family. D indicates the presence of the disease and 1-5 indicate different sized alleles of the linked polymorphic marker. The proband (affected mother) is heterozygous at the disease (D/d) and the linked polymorphic marker locus (alleles 2/4). Marker allele 2 is present in the mother's affected father and her two affected children. The unaffected child has inherited allele 4 from the mother. It can, therefore, be inferred that allele 2 is inherited along with the disease allele (D), i.e. allele 2 is the phase allele. Detection of allele 2 in an embryo would therefore be associated with the presence of the mutated allele.

A combination of multiplex and fluorescent PCR is possible as technological advances have allowed the simultaneous analysis of up to five fluorescent dyes at a time. Products of different size can be differentiated even if labelled with the same fluorescent dye so this further increases the number of primers that can be multiplexed in a single F-PCR reaction.

Finally, it should be noted that other events can also make a heterozygous sample appear as homozygous and give the appearance of ADO, such as chromosomal mosaicism, the presence of haploid cells, mitotic nondisjunction or anaphase lagging by which monosomy can occur (Dreesen et al., 2008). A well-designed multiplex PCR PGD protocol may differentiate between the above and true ADO.

1.1.2.1.3 Contamination

Contamination is a major problem with single cell PCR, mainly because of the large number of cycles (40-65) needed for sufficient amplification from a single genome. Measures to prevent contamination are, for example, separating the single cell PCR set-up and analysis areas, performing PCR in a laminar-flow hood, using dedicated equipment for single cell work, carrying out filtration and testing of reagents as well as UV irradiation of all equipment and consumables prior to use. Apart from extraneous contamination, another source of contamination in PGD comes from sperm or cumulus cells embedded in the zona pellucida (ZP) that can get released during the biopsy procedure. To prevent this, the cumulus cells are removed from around the oocyte and the embryo before biopsy, while intracytoplasmic sperm injection (ICSI) is used for fertilisation instead of standard IVF, to ensure there is no excess sperm present (De Vos and Van Steirteghem, 2001). In addition to these precautions, biopsied cells are usually washed in 2-3 droplets of buffer medium that has been cleared of contamination, before transfer to the PCR tube (Thornhill et al., 2005).

Subsequent to the biopsy and transfer procedures, contamination can also occur during PCR set up. It is, therefore, important that a stringent optimised single cell PCR protocol is run with appropriate positive and negative controls so that any contamination can be detected. Moreover, multiplex PCR PGD protocols involve the simultaneous amplification of polymorphic markers, often referred to as ‘contamination markers’ as they can be used to indicate the presence or absence of contamination (Harper and Wells, 1999). An informative polymorphic marker, one for which all parental alleles are of different size and can easily be differentiated, can be used to determine not only the origin but also the purity of DNA from amplified single cells (figure 1.4).

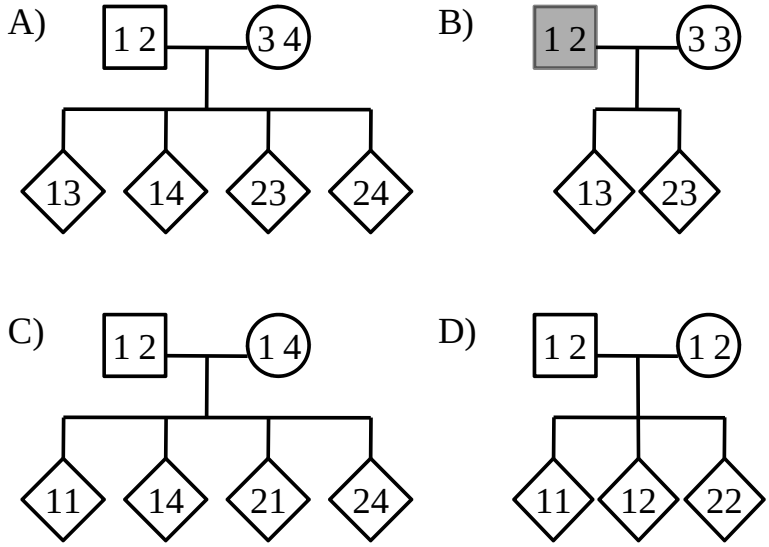

Figure 1. 4: Example of polymorphic marker informativity in four couples, indicating all possible embryo genotypes. The different polymorphic marker allele sizes are indicated by the numbers 1, 2, 3 and 4. A) Fully informative marker. All parental alleles are of different size. B) Informative marker with unaffected partner homozygous for the marker alleles. Maternal and paternal alleles differ. C) Semi-informative polymorphic marker. The couple shares one of the marker alleles and D) Uninformative marker. The couple shares all of their alleles. Polymorphic markers with informativity similar to examples B and C may be of use in a diagnosis in case the marker is linked and the phase allele can be identified, however, in both cases, contamination may not be detected and these markers, should, therefore, be used in conjunction with another fully informative polymorphic marker.

The polymorphic marker can be intragenic, linked, or non-linked to the gene of interest. An intragenic or linked marker can also provide confirmatory results to mutational analysis and, therefore, increase the reliability of the diagnostic strategy (Piyamongkol et al., 2001a;Dhanjal et al., 2007).

Finally, carry-over contamination, the accidental amplification of DNA fragments generated during previous experiments, is also a major source of false positive results. The technique of nested PCR has been used to reduce the risk of carry-over contamination. It involves two sequential amplification reactions, where the larger fragment produced in the first PCR round is used as a template for the second round of amplification. Since a different set of internal (nested) primers is used in the second PCR, the outer first round products cannot get amplified by the second reaction. The two amplification rounds of a nested PCR significantly increase the efficiency of DNA amplification from a single cell. Lower stringency is usually employed during the first

round of PCR and higher stringency in the second round (Wells and Sherlock, 1998) (figure 1.5).

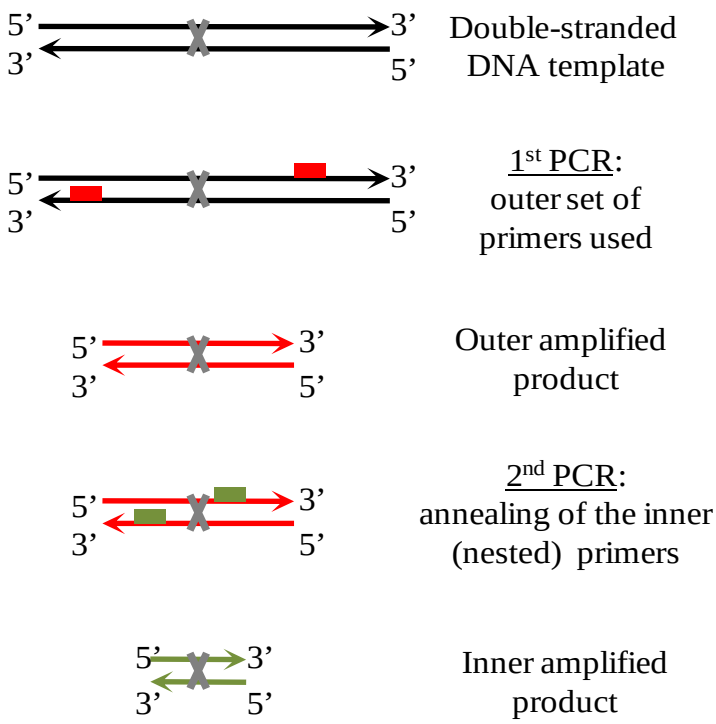

Figure 1. 5: Schematic representation of nested-PCR. The technique involves two sequential amplification reactions. The first amplification reaction employs the outer set of primers to amplify the sample DNA template. An aliquot of the PCR product is taken to set up the second amplification reaction, where the inner set of primers is used. “X” indicates the mutated region.

Contamination is monitored during a PGD case by obtaining and testing negative controls (blanks) from the last wash of each biopsied cell and from the PCR mix to allow testing of the PCR reagents. The general contamination risk may also be assessed during workup by testing tubes that have been left open for some time into the room and hood where the single cell PCR is set up (Thornhill et al., 2005).

1.1.2.2 Mutation detection in PGD for single gene disorders

The ideal PGD protocol needs to be simple, sensitive, quick, accurate, and fairly inexpensive. Several analysis techniques have been applied in PGD for single gene disorders, depending on the nature of the mutation. Size differences are the easiest to detect and are usually analyzed by F-PCR. Other techniques have been used to detect substitutions, deletions or insertions, for example, amplification refractory mutation system (ARMS), restriction fragment length polymorphism (RFLP), single stranded conformational polymorphism (SSCP) and heteroduplex analysis (HA) techniques. A modification of the sequencing technique, known as “mini-sequencing”, has also been performed for direct mutation detection and is becoming common practice in PGD (Ao et al., 1998; Abou-Sleiman et al., 2002; Harper et al., 2002; Bermudez et al., 2003; Fiorentino et al., 2003; Moutou et al., 2003;Moutou et al., 2007). More recently, quantitative real-time PCR and linear after the exponential-PCR (LATE-PCR) have also been used on single cells to allow accurate quantification of the number of copies of an amplicon present in a sample (Rice et al., 2002;Pierce et al., 2003).

Designing, optimising and validating a PGD protocol on single cells can take months. Attempts have been made to establish more universal PGD protocols to bypass the need for development of mutation-specific tests, and decrease the workup times. The linkage strategy, based on detection of the linked markers’ phase alleles, as previously described, is an indirect mutation detection method that can be applied to more than one family affected with mutations in the same gene, even if the causative mutation is different. Unfortunately, the optimisation of multiplex PCR protocols for the co-amplification of a number of markers linked to the disease gene can be very difficult and time-consuming when working with single cells. Whole genome amplification (WGA), which allows non-specific amplification of the entire genome, overall increasing DNA concentration and providing sufficient templates for many subsequent PCR analyses, has been used to overcome these difficulties. Several WGA techniques have been developed and applied on single cells or low amounts of DNA template, for use in preimplantation and non-invasive prenatal diagnosis. Examples include the primer extension preamplification (PEP) method, degenerate oligonucleotide primed PCR (DOP-PCR), tagged random primers PCR (T-PCR), linker- adaptor PCR (LA-PCR), the T7-based linear amplification of DNA (TLAD) and multiple displacement amplification (MDA) (reviewed in Peng et al., 2007, Spits and Sermon, 2009).

PEP was the first WGA method with clinical application in PGD for the diagnosis of a dominant cancer predisposition syndrome, familial adenomatous polyposis coli (Ao et al., 1998). Following that, DOP-PCR and CGH was performed on single polar bodies and blastomeres, while MDA was first applied for PGD for beta-thallasaemia and cystic fibrosis (Wells et al., 2002;Hellani et al., 2005). More recently, MDA was also used as the first step in preimplantation genetic haplotyping (PGH) by linkage analysis, which allows bypassing the need for development of patient specific tests for carriers of the same monogenic disease (Renwick et al., 2006). It should, however, be noted that linkage analysis cannot be applied in cases of *de novo* mutations or when there are no family members available. Testing of single sperm cells or polar bodies has been performed to enable linkage analysis in these couples (Spits et al., 2006). However, even when the phase alleles are known, diagnosis may still not be possible if the couple is uninformative for the polymorphic markers.

According to the most recent report of the ESHRE PGD consortium (data collection VIII), the most common indication for PGD for autosomal dominant diseases is myotonic dystrophy type I (76 cycles), followed by Huntington's disease (HD) (44 cycles, plus 12 cycles for Huntington exclusion testing). In total, ESHRE data collections I-VIII have reported 393 PGD cycles for DM1 and 326 cycles for HD and HD exclusion testing (Goossens et al., 2008b).

1.2 Myotonic dystrophy type I

1.2.1 Clinical characteristics

Myotonic dystrophy type I (DM1, Online Mendelian Inheritance in Man database (OMIM) number 160900) is also known as dystrophia myotonica, myotonia atrophica or Steinert disease. DM1 shows autosomal dominant inheritance and is the most common adult muscular dystrophy. Its prevalence varies between different areas but is generally estimated at 1:8000 worldwide, the highest being 1:500, in the Saguenay-Lac-Saint-Jean region of Quebec, Canada (Meola, 2000;Yotova et al., 2005).

The clinical findings have been categorized into three overlapping phenotypes, the classic form (adult-onset, appearing in late 20s or early 40s, or late-onset, appearing after 40 years of age), the juvenile-onset (appearing after birth and in teen years), or the congenital form (present from birth). The classic form of DM1 was first described in 1909, the disorder was characterized as ‘multisystemic’ in 1936 and the congenital form of DM1 (CDM1) was described in 1960 (Schara and Schoser, 2006). Patients with DM1 present with changes in muscle but in addition, and in contrast to other types of muscular dystrophy, multiple organs and tissues are affected, for example, the eyes, heart, endocrine system, central and peripheral nervous systems, gastrointestinal organs, bone and skin. DM1 manifests with cataracts and myotonia (tonic muscle spasm with delayed relaxation) in the late-onset form, muscle weakness, muscle wasting, cardiomyopathy and cardiac conduction abnormalities, in the juvenile form, and additional baldness, bowel dysmotility, gall stones, or diabetes in the adult-onset form. Infantile hypotonia, respiratory and feeding difficulties, delayed motor and speech development and mental retardation are described in the congenital form. At least 20% of CDM1 infants die in the neonatal period (Edstrom, 1999;Salehi et al., 2007). In the other DM1 forms, sudden death may occur due to the cardiac complications or due to respiratory insufficiency following weakness of the diaphragm (Schara and Schoser, 2006).

DM1 has also been associated with reduced fertility in men, due to testicular atrophy and oligozoospermia (Sarkar et al., 2004). A recent analysis of 44 PGD cycles from 22 couples with DM1 indicated ovarian dysfunction, poor response to ovarian stimulation

and production of lower grade embryos in affected women following ICSI/PGD treatment compared to controls (Feyereisen et al., 2006). Poor response to ovarian stimulation of PGD patients with DM1 was also reported in a subsequent study of 15 PGD cycles from 15 couples with DM1, though no differences in embryo quality and also in oocyte quality compared to controls were observed (Sahu et al., 2008). In contrast to the above, a larger study on DM1 and female fertility (78 couples with DM1, 205 cycles) did not support an impaired gonadal function in the female patients with DM1 undergoing PGD; the authors attributed their findings to the smaller study population of the previous studies (Verpoest et al., 2008).

1.2.2 Molecular characteristics: a triplet repeat disorder

DM1 belongs to the class of triplet repeat disorders. The first trinucleotide repeat expansions were identified in 1991, when the mutations causing fragile X syndrome and spinal and bulbar muscular atrophy were described (Kremer et al., 1991;La Spada et al., 1991;Verkerk et al., 1991). It is now known that tetrameric, pentameric and dodecameric repeats can also expand, associated with myotonic dystrophy type 2, spinocerebellar ataxia type 10 and progressive myoclonus epilepsy respectively, but the largest class of human diseases caused by repetitive element instability involves trinucleotide repeat expansions (Lalioti et al., 1997; Matsuura et al., 2000;Liquori et al., 2001;Gatchel and Zoghbi, 2005). Based on the relative location of the trinucleotide repeat to a gene, triplet repeat disorders can be categorized into two subclasses. The first subclass involves repeat expansion in non-coding sequences, resulting in altered RNA function, whereas the second subclass is characterized by $(CAG)_n$ repeats within gene exons that code for polyglutamine tracts, resulting in altered protein function. The repeats observed in subclass I disorders are usually larger (showing hundreds of triplets) and their exact size is not always known, whereas in subclass II disorders, the repeats are much smaller in number and variation, and repeat sizes fall into well-defined ranges (Cummings and Zoghbi, 2000;Everett and Wood, 2004).

The mutation causing DM1 was identified in 1992, as an expansion of a CTG repeat, mapped on the long arm (q) of chromosome 19 in the 3’ untranslated region of the

myotonic dystrophy protein kinase (*DMPK*) gene, at 19q13.3 (Brook et al., 1992; Fu et al., 1992;Mahadevan et al., 1992;Mahadevan et al., 1993a). The CTG repeat is highly polymorphic and relatively stable within the general population, ranging from 5-37 repeats. Alleles between 38-54 repeats are known as premutation alleles and they are very unstable and liable to frequent expansions. Alleles greater than 55 repeats are associated with disease and are also highly unstable in the germline; they can expand to several hundreds or even thousands of copies. The larger expansions are generally associated with more severe symptoms and an earlier age of onset, evident from generation to generation. This characteristic of trinucleotide repeat disorders is commonly referred to as 'anticipation' and the most striking example of this is of an individual with the classic form of DM1 having a congenitally affected child. Anticipation was first described in 1918 for myotonic dystrophy and has since been observed in all dominant trinucleotide repeat disorders, i.e. except for Friedreich's ataxia (FRDA), which is recessively inherited (Schara and Schoser, 2006).

A second type of myotonic dystrophy was first described in 1994 and the mutation, a CCTG expansion, was mapped in 1998 to the long arm of chromosome 3 (3q21), in intron 1 of the zinc finger protein 9 (*ZNF9*) (Ranum et al., 1998;Ricker et al., 1999; Liquori et al., 2001). The condition was identified as myotonic dystrophy type II (DM2) in 2001 and has a prevalence of 1:1000 (Liquori et al., 2001;Schara and Schoser, 2006).

DM1 and DM2 (OMIM number 602668) both demonstrate an autosomal dominant mode of inheritance, are both the result of a repeat expansion (CTG or CCTG), share many clinical features, for example myotonia and cataracts, but also have some important differences. For example, the severe congenital form and mental retardation have only been reported in DM1. Anticipation has generally only been observed in DM1, although recently the lack of anticipation in DM2 has been questioned as a case of a 2-year old boy born in a family with DM2 history was described. The patient had a 14.5 kb CCTG expansion detected by Southern blotting in *ZNF9*, while expansion of the *DMPK* CTG repeat was excluded. The CCTG expansion was similar to the expansion in the mother, although such comparisons are complicated by the presence of somatic heterogeneity (Kruse et al., 2008). Muscle weakness is predominantly of distal muscles in DM1 and of proximal muscles (body trunk) in DM2 (hence commonly known as PROMM or proximal myotonic myopathy), while muscle pain is a characteristic of DM2. In addition, only few patients with DM2 become severely

disabled by the 6^{th}-8^{th} decade of life, respiratory problems do not normally occur and sudden cardiac death does not seem common in DM2. Finally, repeat size correlates with age of disease presentation in DM1, but not in DM2 (Day et al., 2003;Kurihara, 2005).

Patients with symptoms related to myotonic dystrophy and lacking the DM1 and DM2 mutations have been identified by several investigators. A third locus of myotonic dystrophy has been suggested on chromosome 16p, although this study is still ongoing (Udd et al., 2006). Another unusual multisystemic myotonic dystrophy disorder was described in 2004. Following molecular analysis, no mutations were detected in chromosomes 3 or 19, while a linkage to chromosome 15 was observed. It was suggested that this new type of myotonic dystrophy is referred to as myotonic dystrophy type 3 (DM3), however it was subsequently shown that the reported family had a disease known as inclusion body myopathy with Paget disease and frontotemporal dementia (IBMPFTD) that had not been previously strongly associated with the observed symptoms (Le Ber et al., 2004).

1.2.3 DM1 inheritance

1.2.3.1 *DMPK* haplotype and transmission of the *DMPK* repeat

A 1kb insertion/deletion polymorphism (Alu+/-) located approximately 5kb telomeric to the *DMPK* mutation has been closely associated with the larger repeat alleles, therefore supporting a haplotype founder effect regarding transmission of the *DMPK* alleles (Mahadevan et al., 1993b). In most populations, 5 CTG repeats, the most common repeat allele present in unaffected individuals, are associated with the Alu+ polymorphism allele, 11-13 CTG repeat alleles are associated with the Alu- allele and alleles larger than 19 repeats are associated with the Alu+ allele. Two evolution models have been proposed, one suggesting that 19CTG/Alu+ alleles occurred from expansion of 5CTG/Alu+ alleles and a second one suggesting that both 5CTG/Alu- and 5CTG/Alu+ haplotypes are present, with 5CTG/Alu+ being the most common, and that repeat alleles independently expanded from both haplotypes with a different rate of

expansion (Imbert et al., 1993;Neville et al., 1994;Deka et al., 1996). Analysis of many populations of different ethnic groups has indicated that the prevalence of DM1 in a population correlates well with the frequency of large unaffected repeat sizes detected in the population sample (Pan et al., 2001;Culjkovic et al., 2002;Alfadhli et al., 2004;Acton et al., 2007).

Because of the low reproductive fitness associated with DM1, it has been suggested that preferential transmission of large alleles must be what maintains the frequency of DM1 in the population. Transmission ratio distortion (TRD) may result from meiotic drive, gametic selection or postzygotic viability and has generally been observed in humans, for example, in retinoblastoma, cone-rod retinal dystrophy and several of the trinucleotide repeat disorders, such as dentatorubral-pallidoluysian atrophy (DRPLA), Machado-Joseph disease (MJD), and fragile X mental retardation syndrome (FRAXA) (Evans et al., 1994;Ikeuchi et al., 1996; Riess et al., 1997; Drasinover et al., 2000; Girardet et al., 2000;Zollner et al., 2004).

Several studies have investigated the transmission of the *DMPK* repeat alleles and report segregation distortion of repeat alleles away from Mendelian predictions, though data has generally been contradictive. Early studies of healthy individuals heterozygous for the *DMPK* repeat region supported a hypothesis for meiotic drive and preferential transmission of alleles over 19 repeats mostly by males (Carey et al., 1994;Hurst et al., 1995). Investigations on sperm, however, did not show any evidence for altered segregation patterns or an effect of alleles on sperm viability, though a selective advantage of sperm carrying large alleles during fertilisation remained a possibility (Leeflang et al., 1996). Chakraborty et al., (1996) reanalyzed the previous data and performed additional investigations of transmission to children, overall reporting preferential transmission of large alleles during female, and not male, meiosis. It was also suggested that TRD mechanisms might differ between the different repeat classes. More recently, Dean et al., (2006a) investigated the transmission of normal *DMPK* repeats in preimplantation embryos from general IVF patients and observed TRD in maternal transmissions of large normal alleles and also TRD preferentially to the female embryos.

In addition to the studies on unaffected individuals, other studies focused on the transmission of expanded *DMPK* alleles, reporting preferential transmission of the DM1

expansion (Gennarelli et al., 1994;Zatz et al., 1997;Magee and Hughes, 1998). Contrary to these initial results, however, a subsequent study, which investigated the transmission of mutated alleles from prenatal molecular studies of 83 fetuses from 62 affected mothers and 21 affected fathers, excluded preferential transmission of the expanded allele (Zunz et al., 2004). The findings in the latter study were attributed to the use of prenatal data contrary to pedigree analysis that only investigates offspring that survive to term.

Another prenatal diagnosis study confirmed these results, excluding preferential transmission of the DM1 gene, and also observed a much higher intergenerational expansion when the transmitting parent was the mother compared to when it was the father (Martorell et al., 2007).

1.2.3.2 Repeat instability

1.2.3.2.1 Intergenerational instability

Intergenerational instability has been shown at the level of offspring both for mutant alleles but also for *DMPK* repeats in the normal range (Dean et al., 2006b). Imbert et al., (1993) predicted 19 CTG repeats to be the point at which further instability begins, leading to disease. Expansion of the CTG repeat tract during parent-offspring transmission is thought to occur in two stages. First, a moderate repeat expansion occurs from the normal to the premutation state and this increases the chances that the repeat will further expand to the full mutation state in the next generation (Martorell et al., 2001;Nag, 2003).

For many trinucleotide repeat loci, instability varies depending upon the sex of the transmitting parent. For example, CAG expansion diseases tend to be more unstable on paternal transmission, while in FRAXA, the passage from pre-mutation to full-mutation status occurs most commonly with transmission from the mother and FRAXA males carrying a full mutation in their somatic cells usually transmit a premutated allele to their daughters (Nolin et al., 2003;Zeesman et al., 2004;Wheeler et al., 2007). For DM1, the effect of parental gender on repeat instability varies depending upon the CTG tract

length. For CTG lengths of around 200-600 repeats, a bias for expansion is seen in both paternal and maternal transmissions, while for lengths greater than 600 CTG repeats, there is a bias for maternal transmission of very large (>1000) repeat expansions in a single generation, resulting in the severe CDM1 (Lavedan et al., 1993). Cases of asymptomatic women giving birth to CDM1 children and rare instances of paternally transmitted CDM1 have, however, also been reported (Nakagawa et al., 1994;Die-Smulders et al., 1997;Tanaka et al., 2000).

Contraction of the repeat size during transmission has been reported in DM1 as well as other trinucleotide repeat disorders, for example in FRDA, spinocerebellar ataxia 8 (SCA8) and spinobulbar muscular atrophy (SBMA) (Cleary and Pearson, 2003). In a study of DM1 parent-offspring pairs, the frequency of contraction was 6.4% (data from 1489 DM1 offspring) and was found to be mostly associated with paternal rather than maternal transmissions (Ashizawa et al., 1994). A similar finding was reported in a study of human gametes and embryos, where a larger number of contractions was detected in spermatozoa and embryos from affected males than in oocytes and embryos from affected females (De Temmerman et al., 2004). A complete reduction of the repeat size back to the normal range, referred to as reverse mutation, is rarer than a partial reduction in the repeat. Reversion into the normal range is a male germline specific phenomenon (Monckton et al., 1995). An example of reverse mutation was seen in a case where a 200 repeat expansion from a father affected with DM1, reverted back to a normal size of 30 repeats when transmitted to the child (Amiel et al., 2001).

The above observations have raised several hypotheses, for example that some affected individuals might have a predisposition to reduction during transmission, that a male-specific factor might contribute to repeat deletions, or that there might be a negative selection against sperm carrying the largest expansions.

1.2.3.2.2 Somatic instability

Variability of repeat length also emerges in different cells and tissues of an individual. As a general rule, both the somatic heterogeneity and expansion size increase with age (Wong et al., 1995;Martorell et al., 1998). The rate of instability has been estimated by testing samples from the same individual taken over a period of time. In DM1 the

expansion is thought to be 50-80 repeats per year, contrary to DM2 where a 2000bp expansion was observed over 3 years in an individual (Martorell et al., 1998;Liquori et al., 2001). The level of mosaicism depends on the patient's age, repeat number and type of tissue. In DM1, repeat expansions are found to be smallest in blood and largest in the cardiac muscle, skeletal muscle, brain (except cerebellar cortex), skin and kidney (Monckton et al., 1995;Zatz et al., 1995;Abe, 2002). This tissue-specific somatic instability complicates the evaluations of genotype-phenotype correlation (Marchini et al., 2000). The presence of larger mutant alleles in skeletal muscle, the primary affected tissue, weakly correlates with clinical findings (Zatz et al., 1995). On the other hand, a significant correlation has been found in cases with small expansions between repeat size in lymphocyte DNA and patient's age at time of disease onset (Martorell et al., 1995;Hamshere et al., 1999).

Somatic instability has implications for the analysis of intergenerational transmissions, which are usually investigated by blood DNA analysis of parents and offspring. Careful investigation is needed to distinguish true repeat contractions from cases where the germ-line expansion has been masked by the age-dependent somatic expansion in the parent (Martorell et al., 2000).

1.2.3.2.3 Mechanisms of repeat instability

Suggested mechanisms of repeat instability in DM1 relate to the processes of DNA replication, repair and methylation.

Experiments *in vitro* have shown that repeat sequences are able to form structures comprising both Watson-Crick (WC) and non-WC base pairs, for example hairpins, tetrahelical structures, triplexes or duplexes (Baldi et al., 1999; Mankodi et al., 2000;Heidenfelder et al., 2003;Sarkar et al., 2004;Savouret et al., 2004;Ranum and Day, 2004). CUG repeats, in particular, can form hairpins with U-U mismatches and G-C base pairs that can have an effect during DNA replication, leading to instability. The effect is dependent on the repeat orientation, i.e. whether it is in the template strand or the Okazaki fragment, or its proximity to the replication origin (Yang et al., 2003;Mirkin, 2006). Hairpin formation can lead to both contractions and expansions, either by replication across the hairpin structures or by DNA slippage, respectively (Heidenfelder et al., 2003). DNA slippage causes the DNA polymerase to dissociate and

re-associate with the template strand. During re-association, a misalignment between the template and the newly synthesized strand would generate a single-strand loop that can form a hairpin structure, which is thought to protect the expansion from repair activities of the cell, including mismatch repair and flap endonuclease. The hairpin structures can trap the MSH2/MSH3 dimers of the mismatch repair complex, therefore impairing its ATPase activity and as a result stabilizing the repeats. In addition, the formation of hairpin structures during gap repair DNA synthesis can prevent the function of flap-endonuclease 1 (FEN1), therefore predisposing alleles to further expansion (Mirkin, 2006).

The replication model might explain the differences between male and female transmission of CTG repeats, since, for example, spermatogenesis is characterized by more mitotic cell divisions than oogenesis (Jansen et al., 1994). Expansion during mitosis was also observed in cultured fibroblasts and lymphoblastoid cell lines. Expanded cells of the lymphoblastoid cell lines had a growth advantage over those with smaller expansions or contractions, attributable to increased cell proliferation, but lower survival rate (Zatz et al., 1995;Khajavi et al., 2001). In addition, larger CTG expansions have been observed in tumours of patients with DM1 relative to their nonneoplastic tissue suggesting that expansion can also occur during acquired cell proliferation (Yang et al., 2003). Finally, increased stability in mutants (Fen1/rad27) deficient in Okazaki fragment processing also favors the replication model (Callahan et al., 2003;Yang and Freudenreich, 2007).

Evidence supporting the involvement of the DNA repair mechanism has come from experiments on DM1 mice where it has been shown that the absence of Msh2 shifts the instability from expansions to contractions, both in somatic tissues and through generations. Therefore, in DM1, Msh2 appears to be required for the formation of somatic and intergenerational expansions (Savouret et al., 2003;Savouret et al., 2004). Msh3 and Msh6 deficiency, both partners of Msh2, have the effect of completely blocking the instability or encouraging the expansion respectively (van den Broek et al., 2002). Mouse models have also indicated the mismatch-repair gene, Pms2, as a major component of the expansion pathway; its deficiency decreased the rate of CTG repeat expansion but also increased the frequency of deletions (Gomes-Pereira et al., 2004).

Finally, the involvement of CpG methylation, which is highly regulated in a tissue- and development-specific manner, in repeat instability, restricted to specific loci and tissues and occurring only during specific developmental stages, has also been suggested (Cleary and Pearson, 2003). Methylation status may affect the rate and fidelity of DNA polymerases synthesis, thereby influencing DNA replication and favouring or not the formation of mutagenic intermediates, such as slipped structures (Nichol and Pearson, 2002). DM1 cells with large expansions over 1000 repeats were previously found to be hypermethylated at the CpG island 5' of the CTG repeat (Steinbach et al., 1998). Two CTC-binding factor (CTCF) binding sites flank the CTG repeat forming an insulator unit, which has a role in either blocking enhancers from regulating promoters or protecting from gene silencing. CTCF binding protects the DM1 region from methylation. Expansion in CDM1 is associated with loss of CTCF binding, spread of heterochromatin and regional CpG methylation. Contrary to this, the CpGs of the repeat region are not methylated in classic DM1 (Filippova et al., 2001;Cho et al., 2005).

1.2.3.2.4 Timing of CTG repeat instability

The size of the CTG repeat has been shown to be particularly unstable during spermatogenesis and oogenesis. Male DM1 patients with large repeats in their lymphocytes showed even larger expansions in their mature sperm, while expansion has also been detected in oocytes before completion of the second meiotic division (Abbruzzese et al., 2002;Dean et al., 2006b).

Postzygotically, blastomeres from the same embryo show comparable repeat sizes on analysis but further expansion has been detected to have occurred between day 3 and day 4 in an embryo, though more research is needed to confirm this (Dean et al., 2006b). A pre- and post- zygotic expansion model has also been suggested for another triplet repeat disorder, Fragile X (Florencia et al., 2006).

Expansion is more pronounced during the early embryonic stages. The first wave of somatic instability has been suggested to occur between 13 and 16 weeks of gestation, leading to major heterogeneity between the tissues (Martorell et al., 1997). The second

wave of instability persists throughout adulthood and the remaining life of an affected individual (Martorell et al., 1998).

1.2.4 Mechanisms of DM1 pathogenesis

The first two models of DM1 pathogenesis proposed a mutation effect altering *DMPK* transcription or retaining transcripts in the cell nucleus or causing changes in chromatin configuration, leading to either a decrease in the amount of the *DMPK* protein (*DMPK* haploinsufficiency) or disrupted expression of neighbouring genes, the *SIX5* (homologue of Drosophila sine oculis homeobox 5- *SIX5*) and *DMWD* (dystrophia myotonica-containing WD repeat motif). Though expression of the *SIX5* is suppressed in affected individuals, experiments on Dm15 knock-out mice (the mouse *DMPK* homolog) and six5 knock-out mice, provided evidence for only some of the multisystemic features of DM1, namely skeletal muscle weakness, abnormal cardiac conduction and cataract formation, while *DMPK*-over expressing mice showed skeletal muscle fiber degeneration (Jansen et al., 1996;Berul et al., 1999;Sarkar et al., 2000;Klesert et al., 2000;O'Cochlain et al., 2004;Bates and Gonitel, 2006).

At the same time, the fact that a different repeat mutation (DM2), in a different gene on a different chromosome also caused the predominant clinical features of DM, questioned the DM1 equivalence of both of the above models (Ranum and Day, 2004). Most studies, therefore, have focused on detecting the common pathogenic mechanism between DM1 and DM2 by investigating the potential effect of their common feature, the mutant expanded RNA. Mice expressing mRNA with extended CUG repeats developed myotonia and characteristic DM1 histological features as well as intergenerational repeat instability (Mankodi et al., 2000;Seznec et al., 2001;Gomes-Pereira et al., 2007). Since then, the formation of long hairpin loops by mutant RNA has surfaced as a major factor in DM1 pathogenesis (Sobczak et al., 2003). The expanded CUG repeats are unable to exit the nucleus and as a result accumulate in nuclear foci, where they sequester essential RNA binding proteins, for example CUG binding protein 1 (CUGBP1), muscleblind-like 1 protein (MBNL1) as well as transcription factors (TFs) and prevent their function (Ranum and Cooper, 2006).

CUGBP1 appears to be upregulated in the presence of extended CUG repeats, while two transgenic mouse models of CUGBP1 over-expression develop muscle phenotypes

(Timchenko et al., 2001;Timchenko et al., 2004;Wang et al., 2007). MBNL specifically binds long CUG tracts and colocalizes in vivo with repeats, leading to the depletion and loss of the protein function (Fardaei et al., 2001). Mbnl knock-out mouse models show several features of DM, for example DM-like eye and muscle phenotypes (Kanadia et al., 2003;Hao et al., 2008).

The CUGBP1 overexpression and MBNL loss are responsible for the splicing misregulation of a wide group of developmentally regulated genes in DM1. These include the insulin receptor (IR), cardiac troponin T (c-TNT), muscle chloride channel (CLCN1), MBNL1, the dystrophin gene in skeletal muscle, the microtubule associated protein Tau, the N-methyl-D-aspartate receptor NR1 subunit, the amyloid precursor protein in the brain as well as RYR1, SERCA1, SERCA2 and myotubularin related protein 1 (MTMR1) (Mankodi et al., 2002;Ho et al., 2004;Dansithong et al., 2005;Osborne and Thornton, 2006;Guiraud-Dogan et al., 2007;Nakamori et al., 2007; Wheeler and Thornton, 2007). The disrupted regulation of alternative splicing results in the preferential expression of fetal or neonatal isoforms, inappropriate for a particular tissue (Botta et al., 2008). Overexpression of CUGBP1 has also been suggested to result in binding to inappropriate target mRNAs, enhancing deadenylation and inducing their decay (Moraes et al., 2006).

The binding of TFs by mutant RNA induces their redistribution from chromatin toward a ribonucleoprotein fraction of the nuclear matrix, causing disrupted gene expression patterns, including that of TFs themselves. The multisystemic and multisymptomic nature of DM1 findings can be explained by a common requirement for basic TFs in different tissues. Transcription factor specificity protein-1 (SP1) is one of the TFs most affected by mutant RNA binding (Ebralidze et al., 2004).

Finally, *DMPK* is thought to be indirectly involved in cell cycle checkpoints and chromosome segregation, accounting for the fact that DM1 patients can present with clinical syndromes commonly associated with chromosomal abnormalities, in particular chromosome loss, chromosomal numerical syndromes, or chromosome instability (Rolland et al., 1999;Asano et al., 2000;Casella et al., 2003). It has also been suggested that the *DMPK* expansion might alter some normal functions, such as telomere function, thereby leading to mitotic instability of chromosome 19. This theory is supported by the fact that several of the genes associated with some of the DM1 symptoms, for example

changes in cholesterol metabolism and insulin resistance associated with the low density lipoprotein (LDL) receptor gene or the insulin receptor gene, are located on chromosome 19 (Francke et al., 1984;Yang-Feng et al., 1985).

1.2.5 DM1 management

At present, there is no causative therapy for DM1 and clinical management is aimed at the early detection of complications and treatment of the symptoms. This may involve pulmonary and cardiac investigations, orthopaedic surgery, physiotherapy, management of motor and mental handicaps, cataract and ptosis surgery, pacemaker implantation, or treatment with antimyotonic or anti-diabetic drugs (Kurihara, 2005;Schara and Schoser, 2006). Psychological and pharmacological intervention has also been suggested to DM1 patients for preventing or reducing problems of social isolation, anxiety and depression (Antonini et al., 2006;Laberge et al., 2007).

The extreme variability of the DM1 phenotype, the incidence of anticipation and the impact of the affected parent's sex on repeat transmission, raise difficult issues for genetic counseling, particularly with regards to family planning. The diagnosis of DM1 has a major impact on future pregnancies, offspring as well as other family members. It is often the case that very mildly affected individuals remain subclinically affected and the disease is only diagnosed after the birth of an affected infant, resulting in the parents' families being screened for disease (Fokstuen et al., 2001). On the other hand, individuals with slightly more expanded repeats might not transmit the disease if repeat contraction takes place. The clinical signs, age of onset and family history, along with results from molecular analysis determining a patient's repeat expansion size, all play a role in assessing the risk of having an affected child (Magee et al., 2002). It is worth noting that a familial predisposing effect has been observed where affected sisters give rise to children affected with the same type of the disease (Lavedan et al., 1993).

CDM1 can be suspected prenatally in the late mid- or early third trimester of pregnancy, as certain obstetric complications can be identified sonographically, such as polyhydramnios, talipes, ventriculomegaly or reduced fetal movements. Because these features can be present in other conditions as well, the presence of a DM1 family history

is necessary to support a CDM1 diagnosis. As CDM1 inheritance is mostly associated with maternal transmission, testing for maternal grip myotonia is recommended in these cases, as an additional diagnostic aid in identifying fetuses at risk (Zaki et al., 2007).

Definitive diagnosis during pregnancy is possible by prenatal diagnosis (PND). This is performed by obtaining fetal cells invasively by chorionic villus sampling (CVS) or amniocentesis (AC), and applying PCR protocols to amplify the non-expanded repeat alleles (<100bp) or Southern blotting to allow an estimation of the expanded repeat size (Zuhlke et al., 2000). Less-invasive PND of DM1 has also been reported, where trophoblast cells are retrieved from the lower part of the uterine cavity or foetal DNA is isolated from maternal plasma (Massari et al., 1996;Amicucci et al., 2000).

Because of the unpredictability in DM1 inheritance and the consequent difficulties associated with PND and pregnancy termination, PGD might offer a better reproductive option for DM1 patients.

1.2.6 Preimplantation Genetic Diagnosis for DM1

Due to the very limited amount of DNA obtained from a single cell, routine techniques used for PND, such as Southern blotting for estimation of the repeat size in DM1, cannot be applied to PGD.

The first clinical application of PGD for DM1 was reported in 1997. A hemi-nested PCR protocol was used for amplification of the non-expanded *DMPK* allele from the affected parent, since the expanded allele cannot be amplified by conventional PCR. This protocol could be used for diagnosis of fully informative couples only, where the healthy *DMPK* allele of the affected parent was different from the alleles of the unaffected parent so that both parental alleles could be clearly distinguished in the embryo (Sermon et al., 1997). Following amplification, PCR products were separated on agarose gel. Results indicated a high ADO rate of 24 and 32% on research blastomeres and clinical DM1 cases respectively. This initial hemi-nested PCR protocol was later replaced by a more sensitive fluorescent PCR protocol, also amplifying the

non-expanded *DMPK* alleles, which allowed a statistically significant reduction of ADO to between 5.2 to 6.5% (Sermon et al., 1998a). Following this, and in order to provide PGD for couples that were either non-informative or semi-informative for the *DMPK* region, the same group (Centre for Medical Genetics, Brussels University) optimised, for use at the single cell level, the triplet repeat-primed PCR (TP-PCR) protocol, which had been introduced in 1993 for amplification across expanded repeat regions (Warner et al., 1996;Sermon et al., 2001).

A PGD misdiagnosis occurred when using the first hemi-nested protocol, reported in 1998, where contamination was the possible cause of misdiagnosis in an embryo where only one cell had been analysed (Sermon et al., 1998b). Since then it was decided that diagnosis with protocols based on sole amplification of the *DMPK* repeat region, should incorporate results from two cells from an embryo. This practice is supported by two studies investigating the risk of misdiagnosis in PGD using PCR, proposing that accurate and reliable diagnosis may be possible if results of two genotypes (either two genotypes in a single cell, or a single genotype in two cells) are acquired (Lewis et al., 2001;Navidi and Arnheim, 1991). The incidence of misdiagnosis underlined the importance of using multiplex PCR to allow co-amplification of polymorphic markers along with the mutated region in order to increase the chances of detecting cases of contamination. This led to the development of multiplex PCR protocols for PGD for DM1 (Spits and Sermon, 2009).

The first clinical experience using a multiplex fluorescent PCR protocol, was described in 2001 and aimed not only at reducing the risk of misdiagnosis but also at increasing the diagnosis rate by using a marker linked to the disease gene. Two single-cell PCR protocols were developed where diagnosis was based on co-amplification of *DMPK* with the *APOC2* linked polymorphic marker or with the D21S1414 unlinked polymorphic marker depending on patient informativity for the short tandem repeat (STR) markers (Piyamongkol et al., 2001b;Harper et al., 2002). At the same time, another group reported on the use of two rounds of PCR to allow amplification of the *DMPK* region and one of two closely mapped, highly heterozygous, STRs on chromosome 19, D19S219 and D19S559 (Dean et al., 2001). D19S207 has also been used in a two-round multiplex PCR protocol for PGD for DM1 (Fiorentino et al., 2006).
Aside from improving on the diagnosis of DM1, current research focuses on understanding more about the development and progression of the disease. A main part

of this research has been the identification of characteristic gene expression changes in affected samples. This work has so far mainly involved experiments on affected adult tissue or animal models, as described above. More recently, an embryonic stem cell line was derived from DM1 affected PGD embryos that may be used as a tool to further study the behaviour of CTG repeats (Mateizel et al., 2006). Additional to the stem cell lines, it would be of great interest to further investigate the expression patterns in human embryos during the preimplantation stage of development. A focus on DM1-associated genes may provide some information regarding the impact of the presence of expanded repeats in a cell.

1.3 Investigation of gene expression in preimplantation development

1.3.1 Human oocyte to blastocyst development

The development of the human oocyte occurs in parallel with the development of the surrounding follicular granulosa cell (GC) layers and communication between the two is vital. The GCs comprise of the mural granulosa cells that line the follicle wall, and the cumulus cells that remain in close proximity to the oocyte during growth and following ovulation. The oocyte secretes factors that act on the granulosa cells to induce the expression of genes that regulate all stages of follicle development, and, concurrently, the granulosa cells supply the nutrients that support oocyte growth (Dekel and Beers, 1980;Larsen et al., 1986;Buccione et al., 1990;Zhang et al., 2005). Oocyte-secreted factors include members of the transforming growth factor β (TGF-β) superfamily, for example the bone morphogenic protein- 15 (BMP-15) and growth differentiation factor-9 (GDF-9), that act via downstream SMAD2/3 or SMAD1/5/8 pathways to control cumulus cell lineage and expansion. The impact of these on oocyte growth has been demonstrated by *in vitro* maturation experiments, while the importance of GC-oocyte interactions has been underlined by experiments on mice homozygous for mutations in BMP-15 and GDF-9, which found a phenotype of infertility and defective follicular development (Li et al., 2008). The human oocyte is very active in transcription and translation throughout its growth phase, but becomes transcriptionally inactive late in oogenesis, when it reaches its maximal size (approximately 100μm in the human). Some of the synthesized messenger RNAs (mRNAs) are used for immediate translation, but a large number of them accumulate in the oocyte cytoplasm, stored in an inactive form, and are only recruited when needed at later stages to support maturation, fertilisation and early development (Assou et al., 2006).

During most of folliculogenesis the human oocyte is held in meiotic arrest, at the diplotene stage of the first prophase. Prior to ovulation into the oviduct, following a surge of luteinizing hormone (LH) from the pituitary gland, the oocyte completes the first meiotic division, extruding the small first polar body, and re-arrests at the metaphase stage of the second meiotic division (oocyte maturation). Oocyte maturation is characterized by the disappearance of the large nucleus of the arrested immature oocyte, the germinal vesicle (GV), a process known as GV breakdown (GVBD) to

allow progress into the next steps of meiosis I (MI). A sequence of other events takes place in the MI oocyte, such as chromosome condensation, spindle formation, and separation of homologous chromosomes, overall resulting in the mature meiosis II (MII) oocyte. Apart from the nuclear changes, cytoplasmic changes also take place, including, for example, the maturation of cortical granules, which is responsible for the block to polyspermy (Abbott et al., 1999).

Both meiotic arrest and meiotic maturation are mediated by the communication between the oocyte and the layers of granulosa cells that closely surround it (Corn et al., 2005;Andreu-Vieyra et al., 2006). High levels of LH activate molecular pathways leading to cumulus cell expansion and production of hyaluronic acid (McKenzie et al., 2004;Mehlmann, 2005;Picton et al., 2008). These events are regulated by oocyte-secreted factors that induce the expression of the appropriate cumulus cell genes. The progress from GV to MII is mediated by a decrease in intracellular cAMP in the oocyte, though it still remains unknown exactly how the LH surge stimulates resumption of meiosis, especially since the oocytes lack LH receptors (Kawamura et al., 2004;Jones, 2008).

When the mature oocyte is fertilised, the second meiotic division is completed and the second polar body is released. A series of chromatin modifications guide the formation of two haploid pronuclear masses of equal size, the maternal and paternal, within 3-10 hours post-insemination. In several occasions, during IVF, pronuclei might not be seen in the fertilised oocyte or the oocyte might be abnormally fertilised with a variable number and appearance of pronuclei (Verlinsky and Kuliev, 2000). The pronuclear stage is followed by formation of the zygote, which approximately 20 hours post-insemination starts undergoing mitotic divisions every 12-18 hours (cleavage stage), reaching the morula and eventually the blastocyst stage, of 100-200 blastomeres, prior to implantation. Embryonic transcription is not required for cleavage to occur, as the initial stages of development are dependent on the proteins and transcripts that accumulated in the oocyte during its long developmental arrest in the prophase of meiosis I. In humans, embryonic genome activation (EGA) occurs at the 4-8 cell stage (48-72 hours post-fertilisation), while in the mouse it occurs at the 2-cell stage (24-48 hours) (Braude et al., 1988;Nothias et al., 1995). At the point of EGA the genes that are required for growth and differentiation in the embryo are expressed for the first time. At the blastocyst stage, the cells have differentiated into the outer epithelial trophectoderm

(TE), the surrounding cells that initiate implantation and form extra-embryonic structures, such as the placenta, and a small group of cells called the inner cell mass (ICM), that has the capacity to form all the tissues of the fetus (Duranthon et al., 2008).

Fertilisation and early development are controlled by genetic and epigenetic mechanisms, in which DNA methylation, the addition of methyl groups to cytosine residues, plays a major part. DNA methylation controls the allele-specific expression of imprinted genes, as well as X-chromosome inactivation (lyonization), which occurs at the 10-20 cell stage in humans (Dobson et al., 2004). DNA methylation is mediated by the DNA methyltransferases (DNMTs) including DNA methyltransferase 1 (Dnmt1), the maintenance enzyme responsible for methylation of hemimethylated CpG dinucleotides, and Dnmt3a and Dnmt3b, responsible for *de novo* DNA methylation of unmethylated regions during development. Dnmt1o and the recently identified Dnmt1s, are Dnmt1 isoforms, detected in oocytes and preimplantation embryos (Hirasawa et al., 2008). Another protein, Dnmt3L has no DNMT activity, but colocalizes with Dnmt3a and Dnmt3b and is thought to be essential for establishing methylation imprints in the female germ line (Suetake et al., 2004). Expression of the Dnmt3L gene is essential during murine oogenesis, while in the human transcripts of the DNMT3L gene are only detected after fertilisation, suggesting different imprinting mechanisms between the two species (Huntriss et al., 2004).

The correct pattern of DNA methylation is required for normal mammalian development. In summary, remodelling of the sperm chromatin after fertilisation, which involves removal of protamines and replacement by acetylated histones, is followed by an active demethylation of paternal DNA which is completed before DNA replication. At the same time the maternal genome exhibits a relatively high level of DNA methylation and undergoes *de novo* methylation in human zygotes. During the first cleavages, a passive DNA demethylation of the whole embryonic genome progressively occurs, resulting in a low methylation level at the morula stage, followed by differential *de novo* methylation at the blastocyst stage (Monk et al., 1991;Reik et al., 2001;Fulka et al., 2004).

1.3.2 Gene expression studies

Experiments defining the gene expression profile for each of the stages of preimplantation development, aimed to provide an insight into the molecular pathways that control them. Earlier attempts involved the use of real-time PCR to provide information on gene expression of several genes at a time (Steuerwald et al., 2000;Liss, 2002;Cauffman et al., 2005). Other techniques included serial analysis of gene expression (SAGE) or construction of cDNA libraries and investigation of expressed sequence tags (ESTs) (Adjaye et al., 1997;Neilson et al., 2000; Stanton et al., 2007).
Interesting findings include the abundance of SUMO mRNAs in oocytes, that probably have an important role in both maturation and development, the role of CaSR in the control of meiosis resumption, the association of BRCA1 with chromatin remodeling, and the role of germ specific Y-box protein (MSY2) in the control and translation of maternal mRNAs (Li et al., 2006;Dell'Aquila et al., 2006). The expression pattern of certain genes during development has also given some interesting results. OCT4 is abundantly expressed in the oocyte but its expression varies between blastomeres of the same embryo (Cauffman et al., 2005;Hartshorn et al., 2007). RB1 is low in oocytes and other preimplantation stages but high in blastocysts, potentially indicating a role in apoptosis and differentiation of TE and ICM. Beta-actin (*ACTB*) is high in both oocytes and blastocysts, which might be related to the role in the cytoskeleton, while DNA repair is generally high in the oocytes, decreases during development and increases again in the blastocysts, though different genes seem to function at each of these stages (Wells et al., 2005b;Hamatani et al., 2006).

More recently, microarray technology has enabled the analysis of many thousands of genes from a sample, accelerating the progress of discovery in this area (Bermudez et al., 2004;Dobson et al., 2004;Assou et al., 2006;Kocabas et al., 2006;Li et al., 2006). Because of the small amount of RNA that can be obtained from human oocytes and embryos, 55-100pg from an oocyte and around 20pg from day 3 embryos, an RNA amplification step is required to provide enough RNA for the microarray setup (Neilson et al., 2000;Dobson et al., 2004;Kocabas et al., 2006;Jones et al., 2007). Even though microarray analysis has been performed on individual oocytes following RNA amplification, most work has involved pooling several samples together in order to improve detection of low template mRNA. It has been recommended that a minimum of

three oocytes are pooled prior to RNA extraction and amplification (Jones et al., 2007). If the oocytes are from different donors, this methodology also allows overcoming the individual variation and obtaining a general profile for the tested sample.

Both real-time and microarray results require a kind of normalization prior to analysis, in order to correct for inter-sample variation and other differences involving the quantity and quality of input RNA, efficiency of reverse transcription and amplification processing as well as handling errors. Techniques of normalization include the use of reference genes, RNA mass quantity or exogenous template (Mamo et al., 2007;Mamo et al., 2008). Commonly used reference genes are the so –called housekeeping genes (HKGs), which are responsible for maintaining basic cell functions and are thought to be expressed highly and stably in a variety of tissues. It is important that their expression profile is validated relative to the cell type, stage, or experimental conditions, prior to use for normalization of gene expression results.

Most of the microarray work to date has involved human cumulus cells and oocytes from the GV to MII stage, while limited microarray work has been done on human embryos, as these are more difficult to obtain. Cumulus cells display a very different transcriptome compared to the oocytes. For example, they are more active in cell-to-cell communication, which is in keeping with their very different biological function (Assou et al., 2006). On the other hand, oocytes overexpress genes that are involved in DNA and RNA metabolism, microtubule activity, ubiquitin ligase complex and chromatin modification, as these functions are necessary for the process of meiosis, for remodelling of sperm chromatin upon fertilisation and also metabolizing RNA to support fertilisation and early development (Wassarman and Kinloch, 1992;Kocabas et al., 2006). Other important oocyte pathways are associated with cell maintenance, cell cycle, cell proliferation, apoptosis, energy metabolism and mitochondrial activities (Wells et al., 2005b;Zhang et al., 2005). GVs and MIs have shown similar expression profiles in contrast to MIIs, where many genes are found under- or over-expressed in comparison. This expression pattern, however, may reflect changes due to the completion of meiosis or due to the longer incubation time (Andreu-Vieyra et al., 2006). Finally, investigation of oocytes that failed to fertilize has indicated increased expression of genes for immunological and ribosomal proteins, indicative of shock and defense, elevated inhibin beta-a and beta-b subunits and increased interleukin-1 (Zhang et al., 2005).

The least amount of information is available for human blastocyst stage embryos. It has generally been shown that the blastocyst ICM and the TE express common but also distinct genes and synthesize different proteins, reflecting their ability to differentiate into different cell lines (Adjaye et al., 2005). As indicated in mouse embryos, imprinted X inactivation of the paternal X chromosome occurs in early embryos and is maintained in the trophectoderm, while cells of the ICM undergo reactivation of the paternal X chromosome and random X inactivation (Mak et al., 2004). Mouse blastocysts show increased levels of genes involved in adherens junction, glycolysis and glyconeogenesis and work on human blastocysts has indicated that genes differentially expressed between dormant and activated blastocysts are involved in cell cycle, energy and metabolic pathways, including calcium signalling and adhesion molecules (Hamatani et al., 2006).

Another important aim of the gene expression work has been to identify markers that may indicate oocyte fertilisation potential and embryo development. The selection of best quality oocytes and embryos during IVF, would aim to not only increase the chance of a successful pregnancy, but also move towards single embryo transfer and reduce the incidence of multiple gestations, that are associated with increased fetal morbidity and mortality as well as obstetric complications to the mother (Pinborg et al., 2003;Walker et al., 2004). Findings include the association of pentraxin 3 (PTX3), hyaluronic acid synthase 2 (HAS2) and gremlin 1 (GREM1) cumulus cell transcript levels or of the oocyte BCL2 and proliferating cell nuclear antigen (PCNA) family expression levels with oocyte fertilisation and embryo development. Additionally, the expression of cumulus cell glutathione peroxidase 3 (GPX3), chemokine receptor 4 (CXCR4), cyclin D2 (CCND2) and catenin delta 1 (CTNND1) has been correlated with early-cleavage rates, a marker of predicting pregnancy (Yan et al., 2001;Zhang et al., 2005; Cillo et al., 2007;van Montfoort et al., 2008). More recently, the genes BCL2L11, PCK1 and NFIB, which are also expressed in cumulus cells, were identified as biomarkers for pregnancy outcome (Assou et al., 2008). Markers of DNA damage have also been investigated and abnormal embryo morphologies have been associated with changes in gene expression of several genes, such as TP53 (Wells et al., 2005b;Wells et al., 2005a). Recent advances have also led to the development of methods for proteomic analysis of oocytes and embryos, looking into changes in post-translational events rather than the number of mRNA transcripts (Patrizio et al., 2007).

Further research aiming to determine the expression levels of groups of genes of a certain molecular function or involved in particular biological processes in healthy, normal embryos should enable to define cases where the normal expression is disrupted. This is facilitated by available databases, for example PANTHER or Gene ontology, that provide a description of gene products in terms of the process in which they are involved or their activity at the molecular level, while also allowing an association between the two, as a gene product may be active in one or more biological processes and perform one or more molecular functions (www.pantherdb.org, www.geneontology.org).

Many interesting molecular pathways remain to be investigated in human oocytes and embryos. Expression studies on human embryonic stem cells (hESCs), derived from the ICM of human blastocysts with the potential to differentiate into a variety of specific cell types, might provide interesting data for comparison with preimplantation stage embryos (Thomson et al., 1998). Genes encoding components of the microRNA processing machinery are also of interest. MicroRNAs have been shown to regulate up to one third of human genes by repressing the expression of complementary messenger RNAs, thereby controlling many biological processes in development, differentiation, growth and metabolism (Bartel, 2004;Lewis et al., 2005). Additionally, microRNAs are also known to play a critical role in mouse oocyte maturation and embryo development and have been shown to be implicated in ESC differentiation (Murchison et al., 2007;Tang et al., 2007;Laurent et al., 2008). The expression of genes involved in the microRNA processing pathway has not been previously investigated at the human preimplantation stage.

A comparison of the normal to disease state expression is also possible due to the availability of embryos from PGD patients and may facilitate assessment of disease mechanism for many different disorders.

1.4 Aims of this study

The **first aim** of this study was to identify the most favorable strategies for the development of single-gene PGD protocols, with a focus on the development of protocols for PGD of DM1, aiming to achieve optimal diagnostic efficiency and accuracy. The ultimate aim was to design a universal protocol for the diagnosis of DM1, to reduce time for single-cell optimisation and reduce patient waiting time for treatment. Follow-up analysis of embryos following PGD was performed for all DM1 PGD cycles.

The **second aim** was to investigate the transmission of the *DMPK* repeat in preimplantation embryos. This required initial assessment of the TP-PCR protocol in estimating the size of the CTG repeat allele on genomic DNA, single buccal cells or single lymphocytes, and subsequently on single blastomeres from DM1 PGD cycles. Additionally, analysis of data from diagnosis and follow-up was used to investigate the potential occurrence of transmission ratio distortion, as well as the timing of the expansion, and to detect whether differences exist in preimplantation embryo development of DM1 affected and unaffected embryos.

Finally, the **third aim** was to generate gene expression data and define the molecular portrait of human blastocysts, where limited information is currently available, in comparison to the human oocyte, using microarray technology. The microarray data was used to further investigate the level of expression of genes implicated in specific pathways of interest, in human oocytes and embryo blastocysts.

2. Materials and Methods

An overview of the PGD workflow and general code of practice will be presented at first. Following that, the Methods section has been organized into the following subsections, to describe the procedures associated with each of the aims of this study:

- **Sample collection and preparation**: includes all methods used for PGD-related work, for example nucleic acid extraction or single-cell isolation procedures, as well as collection of samples donated for research from patients undergoing PGD (aims 1 and 2) and collection of samples donated for the expression work (aim 3).
- **Sample processing**: describes all methods used for DNA amplification, including primer design (aims 1 and 2), followed by a description of techniques for RNA isolation, reverse transcription and RNA amplification (aim 3).
- **Sample analysis**: describes the techniques used for analysis of fluorescent-PCR products, together with DNA sequencing, employed for investigation of allele transmission (aims 1 and 2), and concludes with the microarray experiments (aim 3).
- The methods used throughout for **assessment** of sample quality and integrity, as well as details of **statistical analysis** performed, are described last (aims 1-3).

All plastic consumables (tubes, racks, Pasteur pipettes, tips, filter tips) were obtained from VWR International Ltd. The nuclease-free 0.2/0.5ml PCR tubes were obtained from Molecular BioProducts, Inc, UK. Chemicals were purchased by BDH (now merged with VWR International Ltd.) and Sigma-Aldrich, Inc. Enzymes were obtained from Roche Diagnostics Ltd. UK (High Fidelity polymerase and Expand Long Template polymerase) and Applied Biosystems Inc., UK (for Amplitaq Gold). Details for preparation of work solutions and constituency of other reagents are provided in appendix 1 (A1).

2.1 Summary of the PGD procedure

2.1.1 Patient referral

Overall, twenty-three couples at risk of transmitting DM1 to their offspring underwent PGD between June 2004 and June 2008.

The PGD procedure involved an initial consultation with a genetic nurse specialist to provide general counseling on both IVF and PGD procedures, followed by reproductive assessment (semen analysis, assessment of ovarian reserve) to ensure a couple's suitability for treatment. Blood samples were then taken and work-up for the PGD protocol was initiated. Couples received a final "full consultation" with the genetics team and fertility specialists. Any other necessary health tests (for example cardiological assessment) were completed prior to commencing IVF/PGD treatment.

2.1.2 PGD Workup

Five millilitres (ml) of blood was collected in tubes containing 15% ethylene-diamine-tetraacetic acid (EDTA) from the patient, the unaffected partner and affected or unaffected family members when possible. Buccal cell swabs were used to obtain DNA from young children. In some cases, where DNA from previously terminated pregnancies was available, this was also tested to obtain additional information for the PGD work-up for that couple. The genomic DNA extracted from each partner and any relatives was used for the preliminary genetic analysis of the mutated region, along with an assessment for polymorphic markers closely linked to the mutated gene. Each PGD protocol was optimised on genomic DNA and then on single cells, which were either single buccal cells or single lymphocytes. Following optimisation, the final protocol was tested on at least 50 single cells to determine the efficiency of diagnosis prior to clinical application.

2.1.3 IVF/PGD cycle

The IVF treatment was performed at the Assisted Conception Unit, University College London Hospital. In summary, it involved gonadotropin administration to induce the development of multiple follicles as described by Sahu et al., 2008. Ultrasound-guided vaginal oocyte collection was performed at 37 h post-human chorionic gonadotropin (hCG) injection. Oocytes were denuded from their surrounding cumulus cells at 40 h post-hCG, by treating with hyaluronidase, HYASE^{TM}-10x (Vitrolife, UK), according to the manufacturer's instructions. ICSI was performed instead of classical IVF, at 41 h post-hCG, in order to prevent contamination with sperm. Fertilisation was evaluated at 18–20 h post-insemination. Embryos were cultured in IVF medium (GIII series, Vitrolife, UK) and embryo development was evaluated on day 2 and then again on day 3, prior to embryo biopsy. Embryo biopsy was performed in calcium- and magnesium-free biopsy medium (G-PGD, Vitrolife, UK), using either acid Tyrode's solution (in the early PGD cycles) (MediCult (UK) Ltd.) or a 1.48μm diode laser (OCTAX Laser shot™ system, MTG – Medical Technology Vertriebs-GmbH, HUNTER Scientific Limited, UK), for breaching of the zona pellucida, followed by aspiration of the blastomeres through the hole using a biopsy pipette.

Two cells were removed from embryos with six or more blastomeres on day 3, whereas one cell was biopsied from embryos that had five cells or less. If the embryos undergoing protocols that required results from two cells for diagnosis had not grown to at least the six-cell stage by day 3, their biopsy was either deferred to day 4, or proceeded with the removal of one cell; embryos with an unaffected result from this single cell were re-biopsied on day 4 to obtain a second-cell confirmation. Deferred biopsy or re-biopsy, were also generally performed for slow-growing embryos and embryos with inconclusive results. The single biopsied blastomeres were analysed using the already determined patient-specific PGD protocol. If available, a maximum of two unaffected embryos were transferred into the uterus on days 4 or 5 post-insemination and in one case on day 6. Supernumerary unaffected embryos that had reached the blastocyst stage of development were cryopreserved.

2.2 General code of practice

2.2.1 Work-flow

The areas for general laboratory work, nucleic acid isolation, setting up PCR and product analysis were physically separated in order to prevent contamination. The direction of the workflow was always from the main laboratory area to the analysis area. Each of these areas had dedicated pipettes and laboratory coats, as well as reagents and equipment as necessary.

2.2.2 Single cell work

Single cell work practice followed guidelines as described by Thornhill et al., 2005. All single cell work was carried out in a positive pressure room, used only by authorised personnel, which maintained twenty complete air changes per hour to reduce the accumulation of DNA. Designated reagents and other consumables were kept inside the room. Latex or nitrile gloves, hair cover and dedicated disposable lab coats were worn at all times and changed frequently. The room was equipped with a Microflow advanced Bio-safety cabinet class II for setting up single cell PCR. The class II hood was equipped with a UV bulb to allow decontamination of the PCR workspace, pipettes, tips and tubes, at 254nm. Other plastic consumables, cold racks and trays were cleaned with ethanol and exposed to UV irradiation at 254nm in a Template Tamer (Qbiogene, UK) prior to performing PCR. All work surfaces were cleaned with ethanol and dilute bleach weekly and prior to a PGD case. The tubes used for single cell isolation were 0.2ml thin-wall PCR tubes, certified DNA-, DNase-, RNase- and pyrogen-free (Molecular BioProducts, Inc, UK), provided in small bags of 100 pieces to further minimise the risk of contamination. The 0.5ml centrifuge tubes used for PCR setup were also certified DNA-, DNase-, RNase- and pyrogen-free (Molecular BioProducts, Inc, UK).

2.3 Sample collection and preparation

2.3.1 PGD-work

2.3.1.1 DNA extraction protocols

Details of solutions used in DNA extraction are shown in appendix 1, section A1.2.1.

2.3.1.1.1 DNA extraction from blood

DNA extraction was performed as described by Lahiri and Nurnberger, Jr., 1991. Five milliliters of blood were transferred into a 15 ml centrifuge tube and 5 ml of TKM1 were added. The cells were lysed by adding 125 µl of IGEPAL® CA-630 (Sigma-Aldrich chemical company). The mixture was centrifuged at 2500 rpm for 10 minutes (Heraeus Labofuge 400 Benchtop Centrifuge) and the supernatant was carefully discarded. The nuclear pellet was washed in 5 ml of TKM1 buffer, 125 µl of IGEPAL® CA-630 was added again and the tube was centrifuged at 2500 rpm for another 10 minutes (Heraeus Labofuge 400 Benchtop Centrifuge). The TKM1 and IGEPAL® CA-630 washes were repeated until the pellet became white, indicating that all the red blood cells were removed. After the last wash, the supernatant was again discarded. The pellet was gently resuspended in 100µl of TKM1, to which 800 µl of high concentration salt buffer TKM2 and 50 µl of 10% sodium dodecyl sulphate (SDS) were added to lyse the white blood cells. The solution was thoroughly mixed by pipetting up and down several times using a 1ml Pasteur pipette (Alpha Laboratories Limited, UK) and then transferred, using the same pipette, into a 1.5ml microcentrifuge tube. The mixture was incubated at 55°C in a waterbath for approximately 2h until the pellet had completely dissolved and then 300µl of 6 M NaCl were added. The solution was mixed well by tilting the tube several times before centrifuging at 12000 rpm for 5 minutes (MicroCentaur Sanyo MSE). The supernatant was transferred to a new 1.5ml centrifuge tube and the precipitated protein pellet was discarded. Two volumes of 100% ice-cold ethanol were added at room temperature (RT) to the supernatant containing DNA. The tube was inverted several times until the DNA was precipitated and then centrifuged at 1200 rpm for 10mins (MicroCentaur Sanyo MSE). The DNA pellet was resuspended in 1 ml of ice-cold 70% ethanol and the mixture was centrifuged at 12000 rpm for 5

minutes (MicroCentaur Sanyo MSE). The supernatant was discarded and the DNA pellets were air-dried. Each of the pellets was dissolved in 200 µl of 1xTE buffer and stored at 4°C until further use.

2.3.1.1.2 DNA extraction from buccal cells

The buccal cell sample was obtained by gently scraping the inside of the cheek with a sterile mouth swab (VWR, UK) and the cells were suspended in 500µl phosphate buffered saline (PBS) (Sigma Chemical Company, UK) in a 1.5ml microfuge tube. To this, 5µl of 2.6mg/ml recombinant, PCR grade, Proteinase K (PK) (Roche Diagnostics Ltd, UK) and 15µl of 10% SDS (Sigma® Chemical Company, UK) were added and mixed well. Cell lysis was performed by heating at 37°C for 1hr in a waterbath. Following that, the tube was heated at 96°C for 15min in a thermal cycler, in order to inactivate the PK, and 150µl of 6M NaCl were added. After mixing well, the tubes were spun at 12,000 rpm for 5min (MicroCentaur Sanyo MSE). The protein pellet was discarded and a volume of ice-cold 100% ethanol, equal to [2x volume of the supernatant], was added to the supernatant and mixed by inversion. The tube was then centrifuged for 5min at 13,000rpm (MicroCentaur Sanyo MSE). The supernatant was removed and the remaining precipitated DNA was washed in 1ml of ice-cold 70% ethanol. After spinning for 5min at 10,000rpm, the supernatant was removed and the pellet was left to air-dry. DNA was suspended in 50µl TE buffer and stored at 4°C until further use.

2.3.1.2 Isolation of lymphocytes from blood

The lymphocyte isolation procedure was based on a protocol described by Boyum, 1968. Fresh blood was collected in Lithium Heparin tubes. Six millilitres of 0.9% NaCl were mixed with 6ml blood in a 14ml centrifuge tube by inversion. Eight millilitres of the diluted blood was gently pipetted using a Pasteur pipette (Alpha Laboratories Limited, UK) in another tube already containing 6ml of Ficoll-Paque Plus (GE Healthcare Life Sciences, UK) so that the blood formed a layer on top of Ficoll without mixing with it. In order to do that, the tube containing Ficoll was tilted and the blood was slowly

trickled in the side of the tube. Tubes were centrifuged for 30min at 1300rpm at room temperature (Heraeus Labofuge 400 Benchtop Centrifuge). The centrifuge brake was turned off to permit slow deceleration at the end of the centrifugation cycle and allow slow separation of the lymphocytes (figure 2.1). After separation, the lymphocyte layer (buffy coat) was carefully removed using a clean Pasteur pipette and transferred to a clean 14ml tube which was then filled up with 0.9% NaCl. The solution was mixed well and centrifuged, as previously, at 1300rpm for 15 minutes at room temperature with the centrifuge break allowed back on to hasten centrifuge spin-down. The supernatant was discarded and the cell pellet was resuspended in 14ml of 0.9% NaCl and again spun at 1300rpm for 15min with the break on. Washing of the pellet with 0.9% NaCl was performed twice before resuspending in 2ml of 0.9% NaCl. The lymphocyte suspension was stored at 4°C and kept for subsequent isolation of single lymphocytes for up to a maximum of three days.

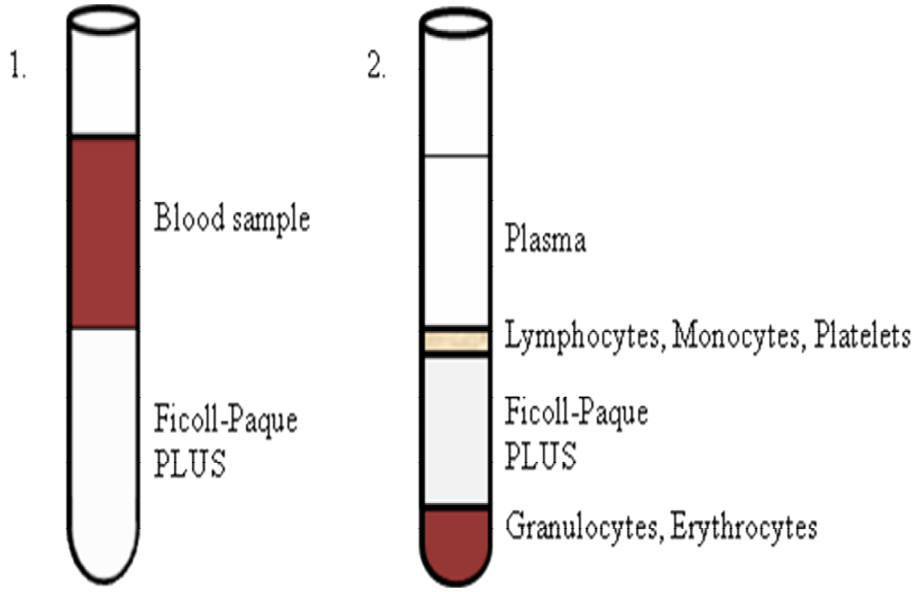

Figure 2. 1: Procedure for isolation of lymphocytes using Ficoll-Paque PLUS (GE Healthcare Life Sciences, UK). 1. Fresh blood, diluted with NaCl, is layered on top of Ficoll and the tube is centrifuged for a short period of time. 2. Differential migration during centrifugation results in the formation of distinct layers containing different cell types. Lymphocytes are found between plasma and Ficoll along with other slowly sedimenting particles. The lymphocyte layer is carefully removed, placed in a clean centrifuge tube and subjected to several washing steps.

2.3.1.3 Isolation of cell clumps and single cells

Buccal cells were obtained as previously described using a sterile mouth swab (VWR, UK) and the cells were suspended in 1ml of calcium- and magnesium-free PBS containing 0.1% w/v polyvinyl alcohol (PVA) (Sigma® Chemical Company, UK). The lymphocyte suspension was isolated from blood as previously described (section 2.3.1.2).

A cell aliquot (buccal cell or lymphocyte) was placed on a Petri dish, on a drop of either PBS/PVA or dissociation buffer (DB) containing 4% bovine serum albumin (BSA) (section A1.2.3), depending on the type of lysis (as described on section 2.3.1.5), (Sterilin, Bibby Sterilin Ltd, UK) and observed under a dissecting microscope (x100 magnification). Another Petri dish containing several 10μl droplets of either PBS/PVA or DB/BSA was also prepared and the cells from the initial aliquot were passed through at least three fresh droplets, using a mouth pipette and a 0.2μm diameter microcapillary (Biohit, UK)**.** In this way, either clumps (3-5 cells) or single cells were isolated. Each clump or single cell was washed at least three times in three clean buffer droplets and then transferred to a 0.2ml PCR tube containing the lysis solution. An aliquot from the last washing droplet was also transferred in another lysis buffer tube to serve as a control.

2.3.1.4 Isolation of single blastomeres for PGD

A new sterile 0.2μm diameter microcapillary (Biohit, UK) was used for blastomere manipulation from each embryo. Each single blastomere was washed in a separate clean petri dish containing droplets of the appropriate wash buffer as previously described (section 2.3.1.3) and then transferred into a transparent 0.2ml microcentrifuge tube containing the lysis buffer.

A small volume of washing medium from the last wash droplet of each single blastomere was transferred in a separate lysis buffer tube and served as a negative control to monitor for PCR contamination.

2.3.1.5 Single cell lysis

Single cell lysis was performed with either PK or an alkaline lysis method following cell isolation as described in section 2.3.1.3. Prior to PK lysis the cells were isolated in droplets of PBS/PVA buffer, while for subsequent alkaline lysis the cells were isolated in DB/BSA buffer. Details of each method are described below.

Proteinase K (PK) lysis: The isolated cells were transferred in 0.2ml sterile thin-walled microcentrifuge tubes containing 3μl of the PK lysis buffer (section A1.2.3). Samples and blanks were incubated at 37°C for 1 hour. Following this, PK was inactivated by incubation at 95°C for 15 minutes. All cells were stored at -80°C for 1.5 hours prior to performing PCR or for up to a few months until further use.

Alkaline lysis: The cells were transferred in 0.2ml thin-walled microcentrifuge tubes containing 2.5 μl of the alkaline lysis buffer (ALB) and kept at -80°C for 1 hour if they were to be used immediately or up to 2 weeks until further use. Subsequently cell lysis was achieved by heating the sample at 65°C for 10minutes. The tubes containing the lysed cells were then transferred to a 0.2ml PCR Cooler (Eppendorf UK Limited) to be kept cold while setting up the PCR. Before PCR amplification the sample was neutralized by adding 2.5μl of 200mM Tricine (Sigma® Chemical Company, Poole, Dorset) into the PCR master mix (Cui et al., 1989).

2.3.2 Collection of samples donated for research from PGD patients

All patients attending the Assisted Conception Unit (ACU), University College London Hospital, were given an information sheet with details on all ongoing research projects. All embryos and oocytes for this project were collected after patients had given their informed consent (Human Fertilisation and Embryology Authority license number RO113). Donated samples were transported from the ACU to the Human Preimplantation Genetics Group and processed immediately (within 30 minutes). Assessment of oocyte maturational status and embryo morphology was performed by

the ACU embryologists. All samples were re-evaluated at the time of processing, to ensure the accuracy of information.

2.3.2.1 Collection of spare embryos following PGD for PCR analysis

Spare embryos following PGD included embryos diagnosed as affected and/or unaffected embryos unsuitable for embryo transfer or cryopreservation. Several drops of either PBS/PVA or DB/BSA were placed on a petri dish, as previously described (section 2.3.1.3), and a drop of acidified Tyrode's solution (MediCult Ltd, UK) was also added to a separate site on the petri dish. Samples were first placed in the drop of Acidified Tyrode's and observed under a dissecting microscope until the zona pellucida had dissolved. The process was facilitated by in- and out- pipetting with the aid of the 0.3µm microcapillary (Biohit, UK), overall taking between 10-30 seconds. On several occasions the blastomeres were easily removed with embryo manipulation from within the zona through the biopsy hole, therefore, use of acid Tyrode's was avoided.

After complete separation of the zona from the embryo, the embryos were then immediately transferred to the clean droplets of wash buffer (PBS/PVA or DB/BSA) on the dish and washed thoroughly as previously described. Embryos were either tubed intact or disaggregated into blastomere clumps or single blastomeres using a 0.2µm microcapillary (Biohit, UK). The capillary was regularly rinsed with wash buffer between each blastomere manipulation.

The single isolated blastomeres, blastomere clumps or whole embryos were transferred in a minimal volume of buffer into individual 0.2ml PCR microcentrifuge tubes containing the lysis buffer. A control blank was taken from the last wash drop as previously described.

2.3.2.2 Collection of cumulus cells and oocytes for PCR analysis

All oocytes were treated with hyaluronidase (HYASE^{TM}-10x supplied by Vitrolife, UK), as previously described (section 2.1.3), to allow their separation from the surrounding cumulus cells prior to ICSI. When needed, these cumulus cells were collected and isolated in clumps or single cells according to the standard procedure. Acidified Tyrode's was used to enable lysis of the oocyte zona.

Donated immature oocytes were separately washed and then transferred to separate microcentrifuge tubes containing PBS/PVA and stored at -80°C until further use.

2.3.3 Collection of samples for gene expression work from general IVF patients

2.3.3.1 Precautions

Every effort was made to maintain strict aseptic techniques throughout all gene expression-related work, i.e. cell isolation, RNA isolation or RNA amplification procedures. Pipettes and tube racks used were designated for RNA work only. All work surfaces and equipment were cleaned with ethanol, then with RNase ZAP (Ambion, Inc., UK), rinsed with distilled water and dried so as to eliminate RNase contamination. Sterile tubes and filtered pipette tips were utilized and gloves were worn at all times and frequently changed. All reactions of the RNA amplification protocol were set up in a Microflow bio-safety cabinet class II.

2.3.3.2 Collection of oocytes and embryos for gene expression analysis

Donated oocytes and embryos were collected in sterile conditions and processed rapidly to minimize RNA degradation. Removal of cumulus cells and the zona pellucida was carefully performed as previously, to prevent contamination with nucleic acids. Samples were rapidly washed in PBS containing 0.1% PVA and 0.3 U/μl of RNasin Plus RNase inhibitor (Promega, UK Ltd). Following washing, oocytes and whole embryos were

transferred to DNA-, DNase-, RNase-free microcentrifuge tubes in approximately 1µl of fluid and then immediately stored at -80°C prior to use.

Fifteen immature human oocytes (MI) were collected from twelve IVF patients with primary or secondary infertility, following patient consent. For four of the couples there was a male factor for infertility (abnormal sperm parameters), while in another couple the female had polycystic ovarian syndrome. There was no known cause for infertility for the remaining seven couples.

All oocytes had matured (MII) in culture and were randomly pooled together in groups of three prior to processing (samples MIIa, MIIb, MIIc, MIId and MIIe). The mean maternal age of all oocytes collected was 36.5yrs±4.47. A more detailed age range for each of the three oocyte sample sets is indicated below:

MIIa: (40+36+36) yrs, MIIb: (36+35+35) yrs, MIIc: (35+40+37) yrs,
MIId: (30+43+39) yrs, MIIe: (25+40+40) yrs.

Similarly, twelve cryopreserved IVF blastocyst stage embryos were thawed following patient consent and pooled together in groups of three prior to processing (samples Ba, Bb, Bc and Bd). Three fresh DM1 affected blastocyst stage embryos, were also donated for research by patient no. 22, affected with DM1, who had undergone two cycles of PGD for DM1. These three DM1 blastocysts were also pooled together (sample DMa).

2.4 Sample processing

2.4.1 Polymerase chain reaction

Polymerase chain reactions were set up on ice in a laminar flow cabinet (Microflow advanced Bio-safety cabinet class II) using dedicated pipettes, sterile pipette filter tips and certified RNase-, DNAse-, DNA- and pyrogen-free microcentrifuge tubes (ABgene, Surrey, UK). Thermal cycling was carried out in Applied Biosystems 0.2 or 0.5ml GeneAmp® PCR System 9700 or the 0.2/0.5ml Eppendorf Mastercycler® Gradient.

For PGD workups, PCR was initially performed using genomic DNA as the template and the genotypes of each family member were determined for the mutated region as well as for several polymorphic markers (linked, such as *APOC2* and D19S112 or unlinked markers). All diagnostic protocols involved direct analysis of the mutated region along with simultaneous amplification in a multiplex PCR of one or two polymorphic markers. Each protocol was first tested on control genomic DNA, and occasionally on diluted genomic DNA (up to a 1/1000 dilution, achieving a concentration of approximately 0.7ng/µl), before being optimised on single cells. The final protocol was tested on at least 50 single cells prior to clinical application in order to determine the percentage of amplification and allele dropout rate (ADO). The percentage of ADO was estimated by counting the number of heterozygote cells whereby only one of the two alleles had amplified, and dividing this by the total number of cells that showed positive amplification. Human blastomeres were also used on two occasions to confirm the single cell amplification efficiencies and allele-dropout rates prior to the clinical application but this practice was stopped, as mentioned in section 4.1.

- Oligonucleotides

Oligonucleotides were ordered from Eurogentec Ltd, UK or Applied Biosystems, UK and supplied as dry pellets, which were reconstituted to a concentration of 50 or 100µM with nuclease-free water (Promega Corp., UK). Details of primer sequences, primer binding sites and product sizes are shown in tables A1.1, A1.2, A1.3 in appendix 1.

Aliquots of the stock solution, 20µl each, were prepared in 0.2ml PCR tubes and diluted to a final concentration of 50µM with nuclease-free water. The pre-aliquoting of the stock solution was done in order to prevent repeat freeze-thawing cycles that would have been necessary if aliquots were prepared from the primer stock on an ad-hoc basis. Working aliquots were stored at -20°C and the remaining primer stock solution was stored at -80°C.

- Oligonucleotide design

Genomic DNA sequences were obtained from the Ensemble Genome Browser website, ensembl release 50 (http://www.ensembl.org/).

The DNA sequence incorporating the mutation or polymorphism was transferred into the Primer3 program via a web interface (http://primer3.sourceforge.net/), and the targeted region was specified. The Primer3 program allowed selection of the best primer

pair for amplification, according to criteria such as primer length (18-30 bases long), primer melting temperature (Tm) (55-65°C) and guanine-cytosine base content (40-60%). Primers were chosen to have minimal self-complementarity and minimal complementarity to each other at their 3'-ends. Specificity of the selected primers was verified using a Basic Local Alignment Search Tool (BLAST) program, BLASTN, in the Ensemble BlastView in order to search for nucleotide sequence similarities against the entire human genomic DNA sequence. The Ensembl database also provided information for the location of single nucleotide polymorphisms (SNPs) in the gene sequence (Ensembl genomic sequence information, transcript information and gene variation info). Oligonucleotide primers were designed to avoid any SNPs within the primer, to prevent interference with primer annealing. SNPs within the PCR product were also avoided, so as not to affect mutation detection or the annealing of any inner primers where required.

The oligonucleotides for amplification of the short tandem repeat (STR) polymorphic markers were obtained using the Ensembl Genome Browser or the GDB Human Genome Database (the latter subsequently shut down on 1st June 2008). Specificity of the STR primer pairs was verified on Ensembl, as above.

2.4.1.1 Standard PCR

The approximate annealing temperatures for the oligonucleotide primers were estimated to be 5°C below the temperature of the primer melting point (Tm). This was calculated using the formula: Tm= 2(A+T) + 4(G+C). The working optimal annealing temperatures were determined empirically by gradient PCR reactions with temperatures either side of the original estimate using the Mastercycler Gradient® thermal cycler. A temperature gradient of ±10°C from the calculated Tm was applied across the block, allowing testing of 12 different temperatures in a single experiment. The temperatures giving the most intense amplified products, as determined by agarose gel electrophoresis, were chosen as the working annealing temperatures.

A variety of conditions were tested during PCR optimisation for PGD protocols. These included testing different cell types (buccal cells/lymphocytes) or lysis conditions (PK/ALB), performing a series of modifications in the PCR reaction components, such

as $MgCl_2$, deoxyribonucleotide triphosphate (dNTP) and primer concentrations or type of enzyme, use of chemical additives in the PCR reaction such as dimethyl sulfoxide (DMSO) and glycerol or modification of the PCR cycling conditions.

A standard PCR mixture consisted of 0.1-0.6µM of each primer, 0.2-0.7mM for each of the dNTPs, (deoxyadenosine triphosphate (dATP), deoxyguanosine triphosphate (dGTP), deoxycytidine triphosphate (dCTP) and deoxythymidine triphosphate (dTTP)), (Promega, Madison, USA), 1x PCR buffer (Amplitaq Buffer, High Fidelity (Hifi) Buffer 2 or Expand Long Template (ELT) Buffer 3), 1.5-4mM $MgCl_2$, 1.25-2 units DNA Polymerase (Amplitaq Gold/High Fidelity or Expand Long Template polymerases), and was made up to a total volume of 24µl with nuclease-free water (Promega, Madison, USA). High Fidelity and Expand Long Template enzymes and buffers were purchased from Roche Diagnostics Ltd. UK. Amplitaq and Amplitaq Gold were purchased from Applied Biosystems Inc., UK.

A PCR “master mix” was prepared to cover the volume of reagents required for all samples and 24µl of the mix were aliquoted into each of the nuclease-free thin-wall 0.2ml PCR tubes. One microlitre of genomic DNA (approximate concentration 0.5-1 µg/µl), was added to each 24µl reaction mix. An extra tube with no DNA was taken as a PCR-mix negative control for each reaction. During PCR set-up for a PGD case two PCR-mix negative controls were made, one after preparation of the PCR master mix and prior to aliquoting the mix into the single-cell tubes, and one at the end of all aliquoting. In cases where contamination was detected the two negative controls provided information on whether the contamination had occurred during cell tubing/PCR set-up or during PCR aliquoting.

PCR for single cells was performed according to the standard PCR protocol, the only difference being that the reagent mixture was made up to a total volume of 21.5µl or 22µl with nuclease-free water (depending on the method of cell lysis, section 2.3.1.5). A volume of 21.5µl or 22µl was added in each of the 0.2ml microcentrifuge tubes already containing the lysed single cells as well as in each of the cell-negative control tubes, containing ~2µl of solution from the last wash drop of each cell. All tubes were centrifuged briefly and then placed in the PCR cycler to start the amplification.

The general conditions of PCR amplification involved an initial denaturation step to activate the enzyme, performed at 94°C for 12 minutes when using Amplitaq Gold polymerase or 95°C for 2 minutes for High Fidelity and Expand Long Template (ELT) polymerases. This was followed by denaturation at 96°C for 15-45 seconds (94°C after the first ten cycles), annealing at 52-65°C for 45s to 1 min and extension at 72°C for 45s to 1 min. Each step was repeated for 15, 40 or 46 cycles depending on the PCR protocol. This was followed by a final extension at 72°C for 5-10min.

The exact PCR conditions for all protocols with clinical application are given in appendix table A2.2.

For other single PCR reactions, the PCR mixture consisted of 0.1μM of each primer, 0.2mM for each of the dNTPs, (Promega, Madison, USA), 1x Hifi buffer, 1.5mM $MgCl_2$, 1.25 units High Fidelity DNA Polymerase and was made up to the required volume with nuclease-free water (Promega, Madison, USA). The following PCR program was performed: 95°C for 2min, (96°C for 15secs, Tm°C for 45secs, 72°C for 45seconds) x 10cycles, (96°C for 15secs, Tm for 45secs, 72°C for 45seconds) x 30 cycles. Melting temperatures for *DMPK*7/8, *ACTB*4/5 and AMELXY primers were 57°C, 62°C, 59°C respectively, and 60°C for other remaining polymorphic markers. Primer details and cycling parameters for detecting expression of β-actin were as described in Salpekar et al., 2001.

2.4.1.2 Multiplex PCR

Multiplex PCR allowed amplification of the mutation marker and one or two polymorphic markers at the same time. The reaction mixture and conditions were similar to those of standard PCR, but more than one set of primers were added in the PCR mixture. The concentration of primers, other PCR reagents and annealing temperatures were modified as necessary during PCR optimisation to achieve optimal amplification efficiency of all targeted regions.

A “split PCR” reaction was performed to allow better overall amplification. This involved an initial multiplex PCR amplification for 15 cycles, followed by a second

round of individual PCR reactions for each of the oligonucleotide sets included in the first round, and performed for an additional 40 cycles. The reaction mixture for second round PCRs was made up to 22µl, to which 3µl of the first round PCR product were added prior to amplification.

2.4.1.3 Fluorescent PCR

Labeling of the forward or the reverse primer used in the amplification reaction with a fluorescent dye, allowed detection of the PCR product on an automated laser fluorescence sequencer (ABI 310, 3100 or 3730 Genetic Analyzer); table 2.1 provides information on commonly used fluorescent dyes. The PCR mixture preparation was the same as for the standard PCR protocol (section 2.4.1.1).

In a multiplex fluorescent PCR, where more than one primer pairs were co-amplified, care was taken to label primers giving an overlapping product size with different fluorescent labels, in order to allow differentiation of the products.

The primer set being amplified under the least favorable conditions in the multiplex reaction, and therefore giving the lowest intensity product, was labeled when possible with a dye of higher fluorescence intensity.

Table 2. 1: Details of absorbance and emission wavelengths for chemical dyes used in fluorescent-PCR. The size of the colour marker in the first column indicates the relative fluorescence intensity (information from Applied Biosystems Inc. and Eurogentec Ltd., www.appliedbiosystems.com, www.eurogentec.com).

Relative Intensity	Dye Chemical Name		Absorbance maximum	Emission maximum
	(6, 5) FAM™	Fluorescein, derivatized as NHS ester via a carboxyl at position 5 or 6	495nm	520nm
	HEX™	Hexachlorofluorescein, NHS ester. Can only be used on 5' end of oligo	535nm	555nm
	JOE™	6-carboxyl-4',5'-dichloro-2',7'-dimethoxyfluorecein, NHS ester	529nm	555nm
	ROX™	Carboxy X-rhodamine, NHS ester	588nm	608nm
	TAMRA™	Carboxy tetramethyl rhodamine, available as NHS ester, or direct linked	559nm	583nm
	TET™	Tetramethyl fluorescein. NHS ester. Can only be used on 5' end of oligo.	522nm	539nm
	NED™	ABI proprietary "yellow"	553nm	575nm
	Dragonfly Orange™	Eurogentec Ltd, UK alternative to NED	554nm	576nm
	VIC®	ABI proprietary "green". Same emission wavelength as JOE, but narrower spectral peak and brighter signal	538nm	554nm
	Yakima Yellow®	Eurogentec Ltd, UK, alternative to VIC	530.5nm	549nm
	PET™	ABI proprietary "red"	558nm	595nm
	LIZ™	ABI proprietary "orange"	638nm	655nm

2.4.1.4 TP-PCR

The triplet primed PCR, specifically used for the amplification across the CTG repeat of the *DMPK* gene, was carried out according to Warner et al., (1996) with some modifications. Primer P2 was a fluorescently-labeled primer complementary to a sequence close to the CTG repeat that was used in combination with a pair of primers, P4CAG and P3R. P4CAG consisted of a 3'-end sequence containing a tandem repeat of 5 CAG trinucleotides, and a 5' tail of 21bp with no homology to the human genome. Primer P3R was only represented by the 21bp sequence of primer P4CAG (figure 2.2).

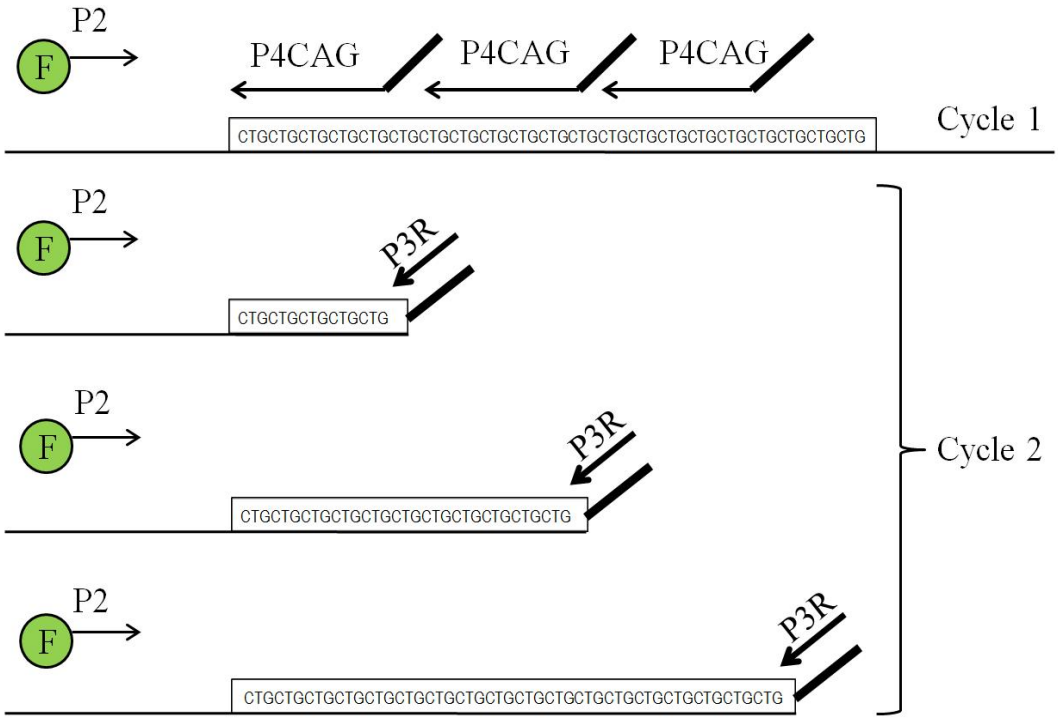

Figure 2. 2: Primers of the TP-PCR method (P2, P3R and P4CAG) used for amplification along the expanded region. Primer P3R was complementary to the 21bp unique sequence of primer P4CAG (black bar).

2.4.1.5 Whole-Genome Amplification

Whole genome amplification (WGA) was performed by isothermal strand displacement using the GenomiPhi HY DNA amplification kit (GE Healthcare UK Limited) for confirmation of diagnosis in some of the PGD spare embryos. Following blastomere isolation in DTT/KOH lysis buffer (appendix section A1.2.3), DNA was briefly denatured by heating to 95°C for 3 minutes, and then cooled to 4°C in buffer containing random hexamers that non-specifically bind to the DNA. A master-mix containing Phi29 DNA polymerase, additional random hexamers, nucleotides, salts and buffers was added and isothermal amplification was performed at 30°C for 6 hours. Following amplification, the enzyme was heat-inactivated by incubating at 65°C for 10 minutes, in order to prevent degradation of the amplification product. Amplification products were stored at -20°C.

The WGA products were tested for the amplification of the *DMPK* triplet repeat region, *APOC2*, D19S112 and AMELXY polymorphic markers (appendix table A1.1).

2.4.2 Processing of samples for gene expression analysis

2.4.2.1 RNA extraction

Total RNA was isolated from clumps of lymphocytes, single oocytes, and whole embryos using either the Absolutely RNA NanoPrep Kit (Stratagene- Agilent Technologies UK Limited) or the AllPrep DNA/RNA Micro kit (Qiagen Ltd., UK), according to the manufacturer's instructions. Centrifugation steps were performed using a Hettich® Mikro^{TM} 200R benchtop refrigerated centrifuge.

The Absolutely RNA NanoPrep Kit was used for RNA isolation during optimisation of the RNA extraction technique from single cells. In summary, the Absolutely RNA NanoPrep Kit protocol (Stratagene, UK) employs a lysis buffer that contains a strong protein denaturant, the chaotropic salt guanidine thiocyanate, to lyse cells and prevent RNA degradation by ribonucleases (RNases). Following cell lysis, the sample was transferred to an RNA-binding nano-spin cup sitting within a 2-ml collection tube to enable the RNA to bind to a silica-based fiber matrix. A DNase digestion step, performed at 37°C for 15 minutes, removed contaminating DNA and then a series of washes removed the DNase and other proteins. Lastly, 10μl of elution buffer (low-ionic-strength buffer), pre-warmed to 60°C, was added directly onto the fiber matrix inside the spin cup and the sample was incubated at room temperature for 2 minutes. The purified RNA was eluted in the collection tube by centrifugation at ≥ 12,000x g for 5 minutes, then transferred to a 0.2ml microcentrifuge tube and stored at -20°C for up to one month or at -80°C for long-term storage.

RNA was extracted from all samples used for microarray analysis using the AllPrep DNA/RNA Micro kit (Qiagen Ltd., UK). Oocytes and blastocyst-stage embryos from different patients were randomly pooled together in groups of three, prior to RNA

extraction. Each set of samples was first lysed in a highly denaturing guanidine-isothiocyanate–containing buffer in order to inactivate DNases and RNases and ensure isolation of intact DNA and RNA. The lysate was first passed through an AllPrep DNA spin column that binds genomic DNA of an average length of 15-30kb. Ethanol was added to the flow-through from the AllPrep DNA spin column to provide appropriate binding conditions for RNA. The samples were then applied to an RNeasy MinElute spin column, where total RNA, longer than 200 nucleotides, was bound to the membrane, allowing contaminants to be efficiently washed away. High-quality RNA was eluted in approximately 14 µl water. This procedure allowed RNAs smaller than 200 nucleotides, (such as 5.8S rRNA, 5S rRNA, and tRNAs, which together comprise 15–20% of total RNA) to be selectively excluded, therefore, providing an enrichment for mRNA. Despite the limited starting material, no carrier RNA was used during the purification process, in order to escape interference with the reverse transcription step and oligo-dT primers of the amplification procedure.

2.4.2.2 Reverse transcription

Reverse transcription was performed following RNA extraction with the Absolutely RNA NanoPrep Kit (Stratagene- Agilent Technologies UK Limited) for initial single-cell RNA work practice.

Five microlitres of each RNA sample (extracted with the Absolutely RNA NanoPrep Kit) was mixed in a 0.2ml microcentrifuge tube with 1µl 10mM dNTPs (Promega, UK), 1µl random hexamers (50ng/µl) (Superscript III First Strand Synthesis System for RT-PCR, Invitrogen) and nuclease-free water to make final volume up to 10µl. The mixture was incubated at 65°C for 5 minutes and then placed on ice for at least one minute. 10µl of cDNA synthesis mix was then prepared for each reaction, comprising of 2µl 10x RT buffer (Superscript III First Strand Synthesis System for RT-PCR, Invitrogen Ltd., UK), 4µl 25mM MgCl2, 2µl DL-Dithiothreitol (DTT) (0.1M), 1µl RNaseOUT (40 U/µl) and 1µl Superscript III RT (200U/µl; Superscript III First Strand Synthesis System for RT-PCR, Invitrogen Ltd., UK). This was added to each RNA/primer mixture, mixed gently, collected by brief centrifugation and then incubated at 25°C for 10 minutes followed by 50 minutes at 50°C. The reaction was terminated by incubating at 85°C for 5 minutes

and then placing on ice. The reactions were collected by brief centrifugation. 1µl RNase H (Invitrogen Ltd., UK) was added to each tube followed by incubation at 37°C for 20 minutes. The resultant cDNA samples were stored at -20°C or used for PCR immediately.

The primers for cDNA amplification were designed to span one intron of each genomic sequence tested, in particular intron 4-5 of the *ACTB* gene and intron 7-8 of the *DMPK* gene. In this way, genomic contamination could be detected by giving a larger PCR product size than the product generated from the cDNA. Primer details are provided in the appendix table A1.3. Exon/intron positions were confirmed using the Ensembl database, release 53 (*ACTB* Ensembl transcript ID ENST00000331789 and *DMPK* Ensembl transcript ID ENST00000291270).

Total HeLa RNA (provided in the Superscript III First Strand Synthesis System for RT-PCR, Invitrogen Ltd. UK) was used as a positive control to confirm successful reverse transcription and cDNA amplification. Two negative controls were tested alongside each amplification; an RNA negative control, which contained HeLa RNA with no reverse transcriptase enzyme, and a PCR negative control, containing all PCR reagents with no cDNA. Nuclease-free water was added instead of RNA/cDNA in the negative control samples.

2.4.2.3 RNA amplification

RNA extracted with the AllPrep DNA/RNA Micro kit (Qiagen Ltd., UK) was amplified in a two-round *in vitro* transcription procedure and converted into digoxigenin (DIG)-labeled complementary RNA (cRNA), using the NanoAmp RT-IVT Labeling kit (Applied Biosystems,UK) according to the company-provided protocol (figure 2.3). Each round of amplification was completed within 6 hours, followed by an overnight incubation step and the two rounds were completed within three consecutive days (~15 hours). Three to four samples were processed at a time. All reagents, apart from the enzymes, were vortexed using Vortex Genie-2 Scientific Industries Inc (VWR international, UK) and briefly centrifuged prior to use (MicroCentaur Sanyo MSE).

Standard laboratory practices for RNA-work were followed (section 2.3.3.1) and nuclease-free tips and reagents were used throughout. All steps requiring incubation at

temperatures between 25ºC and 70ºC were carried out in the GeneAmp® PCR System 9700 (Applied Biosystems, UK) thermal cycler.

The first round of amplification involved reverse transcription using T7-Oligo (dT) primers to make single-stranded complementary DNA (cDNA). This was performed at 25°C for 10 minutes, 42°C for 2 hours, followed by 5 minutes at 70°C to inactivate the reverse transcription enzyme (NanoAmp RT-IVT Labeling kit, Applied Biosystems, UK) and an indefinite hold at 4°C. The reaction tube (20µl cDNA mixture) was kept cold on a 0.2ml PCR Cooler (Eppendorf UK Limited), while the components for setting up the first-round second-strand synthesis were added to make a final volume of 100µl. The second-strand synthesis reaction was performed at 16°C for 2 hours followed by 5 minutes at 70°C for enzyme inactivation as before. The double stranded cDNA was purified using the DNA purification columns provided with the kit, according to the protocol provided. The next step involved an *in vitro* transcription (IVT) labelling reaction, which was set up at room temperature by adding 4µl of each of the reaction components (10x IVT Buffer, NTP mix, IVT enzyme mix) to the cDNA output and performed overnight, at 37°C for 9 hours to obtain cRNA.

The cRNA was purified using the RNA purification columns and eluted in 100µl of nuclease-free water in a RNA collection tube. The purified cRNA was concentrated by vacuum centrifugation (Concentrator plus, Eppendorf Limited, UK), using the 4°C temperature setting, to achieve a final volume of approximately 10µl.

The entire volume of concentrated purified cRNA (10µl) was used in the second round of amplification, similarly comprising of reverse-transcription, second-strand synthesis, cDNA purification and second-round IVT labelling (overnight reaction). During the second round of amplification, Digoxigenin-11-uridine-5'-triphosphate solution (DIG-UTP) (Roche Molecular Biochemicals) was incorporated into the IVT labelling reaction, permitting subsequent chemiluminescent detection after hybridization to a Human Genome Survey Microarray v2.0 (Applied Biosystems, UK). The amplified labelled cRNA was purified and its integrity and concentration were assessed as described in section 2.5.4.

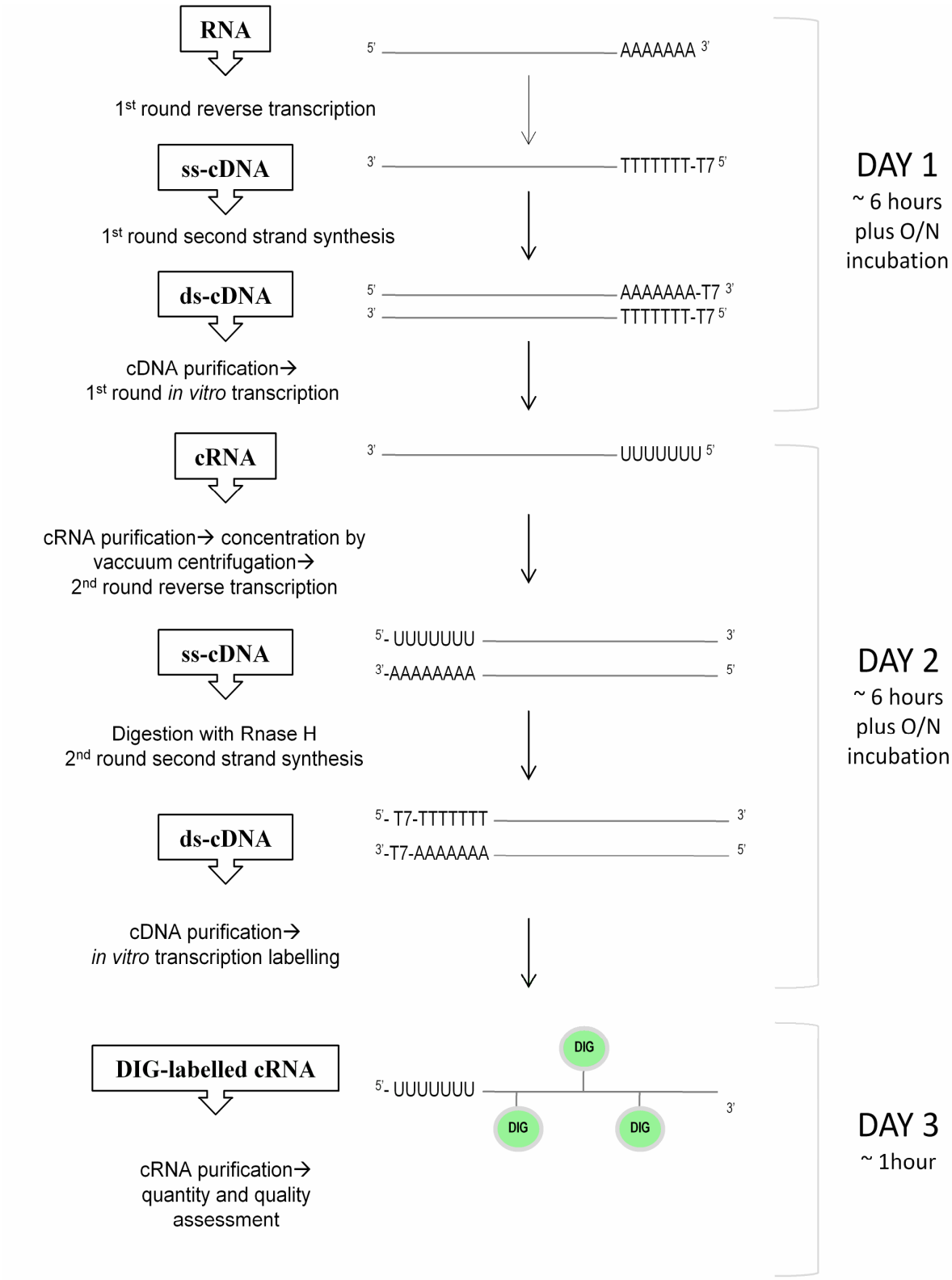

Figure 2. 3: Summary of the Nano-Amp RT-IVT labelling protocol for RNA *in vitro* transcription and amplification. Two rounds of reverse transcription, second strand synthesis and *in vitro* transcription were completed. The 1st round cRNA was concentrated using speed vac centrifugation so that the whole amount could be used in the 2nd round of amplification (Concentrator plus, Eppendorf UK Limited). Labeled nucleotides were incorporated into the RNA during the second amplification step to allow detection following hybridization on the microarrays.
ss-cDNA: single stranded complementary DNA. ds: double-stranded, O/N: overnight

2.5 Product analysis

2.5.1 Analysis of fluorescent PCR (F-PCR) products

The genomic-DNA F-PCR amplification products were diluted to a ratio 1:10 with nuclease-free water prior to fragment analysis. This was done in order to prevent the appearance of artifactual peaks and corruption of the automatic sizing and analysis that are observed with very strong fluorescent signals. The control DNA included in every PCR, not only served as a positive control for the PCR amplification but also allowed monitoring of sizing precision during analysis.

2.5.1.1 F-PCR analysis using ABI Prism 310

A mixture of 1.5µl diluted fluorescent PCR product, 12µl Hi-Di Formamide (Applied Biosystems, UK) and 0.5µl of Genescan™ 500 TAMRA, Genescan™ 350 TAMRA or Genescan™ 500 ROX size standard (PE Applied Biosystems, Warrington, UK) was prepared in a 0.5ml genetic analyzer sample tube (Applied Biosystems, Warrington, UK). Hi-Di formamide was frozen in aliquots at -20°C to prevent degradation due to multiple freeze-thawing cycles. The sample tube was capped with rubber septa (the rubber septum was squeezed to make sure the hole of the lid that the genetic analyzer capillary goes through for sample collection, was open). Samples were denatured at 95°C for 5 minutes and kept on ice for a short time prior to loading on to a 48-tube autosampler tray of the ABI Prism™310 sequencer (PE Applied Biosystems, Warrington, UK). Samples were injected into the single 30cm capillary for 5 sec at 15,000 V. Separations were performed at 15,000V for 24min with a run temperature of 60^{o}C using 310 Genetic Analyzer performance optimised polymer POP-6™ (Applied Biosystems, UK) and 1x Genetic Analyzer Buffer with EDTA. Two matrix standard sets were used to generate a spectral matrix for the following dye sets 6'-FAM™, HEX™, TET™ and TAMRA™ or 6'-FAM™, NED™, VIC® and ROX™.

2.5.1.2 F-PCR analysis using ABI Prism® 3100/3730 Genetic Analyzers

The samples were prepared for analysis in a MicroAmp™ Optical 96-Well Reaction Plate (Applied Biosystems, UK). Similar to the preparation of samples for the ABI Prism® 310, 1μl of diluted fluorescent PCR product was added to 12μl of Hi-Di Formamide (Applied Biosystems, UK) and 0.5μl of a size standard, either Genescan™ 500 ROX for use with the ABI Prism® 3100 or Genescan™ 500 LIZ for the ABI Prism® 3730. Samples were injected onto the ABI 3100 16-capillary array for 5 sec at 1,000V or onto the ABI 3730 96-capillary array for 5 sec at 2,000V. Separations were performed at 15,000V for 22 min, with a run temperature of 60°C, using the POP-6™ sieving polymer for 3100 or POP-7™ polymer for the 3730, 1x genetic analyzer buffer with EDTA and a 36cm array. The data was analysed using Genemapper analysis software version 3.5 (PE Applied Biosystems, Warrington, UK). The matrix standard set was used to generate a spectral matrix for the five dyes 5'-FAM™, NED™ or Yakima Yellow®, VIC® or Dragonfly Orange™, PET™ and ROX™.

2.5.2 Preparation of PCR amplified template for DNA sequencing

DNA sequencing allowed sizing of the CTG repeat on the non-expanded *DMPK* allele. Primers *DMPK*2 and *DMPK3* were used to amplify the *DMPK* repeat region, at a concentration of 0.2μM each in a standard PCR using High Fidelity polymerase (Roche applied science, UK). The PCR product was purified using Centricon®-100 columns (Applied Biosystems, UK) and the DNA quality was assessed on an agarose gel. The sequencing reaction was set up in 20μl reactions containing 0.15μM of either *DMPK2* or *DMPK3* sequencing primer, 0.5x BigDye® Terminator sequencing buffer (5x), 0.5x BigDye® Terminator ready reaction mix, with up to 10ng of PCR product according to the BigDye® Terminator v1.1 sequencing kit (Applied Biosystems, UK) protocol. The sequencing reaction program involved an initial denaturation at 96°C for 1min, followed by 25 cycles of [96°C for 10 seconds, 50°C for 5 seconds, 60°C for 4 minutes]. Extension products were purified following an Ethanol/EDTA/sodium acetate precipitation method according to protocol, in order to remove unincorporated dye

terminators prior to electrophoresis. Each sample pellet was dried by heating at 37°C for 5-15 minutes, then resuspended in 10µl Hi-Di™ Formamide (Applied Biosystems, UK) and transferred onto a MicroAmp® 96-well reaction plate (Applied Biosystems, UK). Sample electrophoresis was performed on the ABI Prism® 3100 Genetic Analyzer using POP-6™ polymer.

2.5.3 Microarray analysis

Eight microarrays were run with human MII oocytes and human blastocyst-stage embryos. A highly sensitive microarray platform was employed, the Applied Biosystems Human Genome Survey Microarray v2.0, which has 32,878 60-mer oligonucleotide probes for the interrogation of 29,098 genes. Prior to hybridization, the labelled cRNA product was fragmented by mixing 90µl of 5 or 10µg cRNA product and nuclease free water with 10µl of cRNA fragmentation buffer (Chemiluminescence detection kit, Applied Biosystems) and incubating at 60°C for 30 minutes. The reaction was neutralized by adding 50 µl of the cRNA Fragmentation Stop Buffer from the kit and keeping on ice. Hybridization of the sample to the microarray, antibody binding and the chemiluminescent reaction were performed as described in the Applied Biosystems Chemiluminescent detection kit. Detection of chemiluminescence, image capture and processing was performed on the Applied Biosystems 1700 Chemiluminescent Microarray Analyzer (Expression Array System Software v1.1.1). Microarray hybridization and image acquisition was carried out by Dr Paul Smyth (Molecular Histopathology laboratory, Trinity Centre for Health Sciences, Dublin, Ireland).

2.5.4 Sample assessment

2.5.4.1 DNA and RNA agarose gel electrophoresis

A 2% agarose gel was prepared by heating 1g of agarose type I (Sigma® Chemical Company, Poole, Dorset) in 50ml 1xTBE (appendix 1, section A1.2.4). The mixture was brought to boiling using a microwave oven at medium intensity for 1-2 minutes with 30 second intervals to mix until the agarose was dissolved completely. The

dissolved solution was left to cool, then 8-10µl of 10mg/ml ethidium bromide (Sigma® Chemical Company, Poole, Dorset) was added into it, and it was subsequently poured into a mini gel tank with a 16-well gel slot former and left to set for 30 minutes at room temperature. The stoppers and comb were then carefully removed and 50ml of 1xTBE added to the electrophoresis apparatus.

A 5-10µl aliquot of each PCR product, of negative control or 2µl of labelled cRNA was mixed with 1-2µl of agarose gel loading buffer (appendix 1, section A1.2.4) and loaded into the well slots. A DNA molecular weight marker, HyperLadder VI, (Bioline Research) or an RNA molecular weight marker, 0.5-10kb RNA Ladder (Invitrogen Ltd, UK) was loaded alongside the PCR products or labeled cRNA to allow evaluation of the DNA/RNA fragment size respectively. The RNA ladder was denatured at 72°C for 10 minutes prior to loading. Electrophoresis was performed at 60 Volts for approximately 30 minutes. The gels were examined under a UV transilluminator (Alpha Innotech Corporation, MultiImage Light Cabinet, Flowgen Staffordshire).

2.5.4.2 Assessment and analysis of whole genome amplification products

The range of DNA fragment sizes produced following WGA was determined by electrophoresis on a 1% agarose gel stained with ethidium bromide as previously described (section 2.5.4.1). Electrophoresis also revealed whether or not any of the degenerate or semi-degenerate primers used had preferential annealing sites within the genome, indicated by distinct bands on the gel. The intensity of fluorescence observed on the gels was an indication of the amount of amplification.

2.5.4.3 NanoDrop® ND-1000 Spectrophotometer

1.2µl of the labeled cRNA sample was loaded on the NanoDrop® ND-1000 Spectrophotometer, providing accurate concentration readings (ng/µl) as well as a 260/280 and a 260/230 nm ratio giving further information on cRNA quality. RNA samples were considered of good quality for further analysis only if the absorbance ratio at wavelengths of 260/280 nm was greater than 1.8, indicative of little DNA contamination. Samples with a lower than 1.8 260/280 ratio may indicate the presence

of protein, phenol or other contaminants that absorb strongly at or near 280nm. 260/280 ratios over 2.1 indicate the presence of degraded RNA, truncated cRNA transcripts and/or excess free nucleotides. In addition, samples with a low 260/230 ratio (less than 1.8) were avoided as the low ratio indicates a significant presence of the reagents used in RNA extraction, which absorb light at 230nm wavelength, and would interfere with the downstream processes.

2.5.4.4 Agilent Bioanalyzer 2100

The quality and size of each labelled cRNA, as well as the absence of DNA contamination or RNA degradation, were verified using the Agilent bioanalyzer 2100 (Agilent Technologies UK Limited). Extracted RNA from all samples prior to RNA amplification was tested by analyzing on a Eukaryote Total RNA Pico Series II chip, while amplified RNA was tested on a Eukaryote Total RNA Nano Series II chip. The RNA concentration, rRNA ratio (28s/18s) and RNA Integrity Number (RIN) were recorded. An rRNA ratio greater than 2.0 indicates little RNA degradation. The RIN number is assigned by the Agilent Bioanalyzer software to assess RNA quality in terms of degradation and allow comparison between samples. It is based on a numbering system from 1 to 10, with 1 being the most degraded profile and 10 being the most intact (Schroeder et al., 2006).

2.5.5 Statistical and microarray analysis

The binomial test and Fisher's exact test were mainly used for analysis of data from PGD cases, as indicated in the results sections 3.1 and 3.2. Values of $p < 0.05$ were considered statistically significant.

For the expression work, analysis of data was performed using Spotfire© Application Package for Data Analysis of the Applied Biosystems 1700 Chemiluminescence Expression System. Signals from each microarray were subjected to quantile normalization and filtered for signal to noise ratios < 3 and flags over 5000 (detectability filter). After normalization, the data for each gene were reported as a logarithm of the expression ratio, i.e the normalized value of the expression level of the

gene divided by the normalized value of the control. The experimental factor of the analysis was the cell type. Data from blastocyst embryos was compared to the data from the MII oocytes, which were used as controls. Relative fold changes were calculated for comparison of individual samples. A t-test analysis was performed and genes were characterized as differentially expressed for p-values <0.05 (the initial array analysis was performed by Dr Paul Smyth).
A dendogram was produced with agglomerative hierarchical clustering, to allow visual interpretation of the results and identify genes showing similar patterns of expression.

Additionally, data from each gene was reported as a logarithmic value (log_2) of the expression ratio (log_2(ratio)), i.e. the ratio of the expression level of the particular gene in the test sample (blastocyst) divided by its value for the control. According to this, a gene upregulated by a factor of 2 (ratio=2) would have a log_2 (2) of 1, whereas a gene downregulated by a factor of 2 (ratio=0.5) has a log_2 (0.5) of -1. Genes expressed at a constant level (ratio=1), would have a log_2 (1) of zero. An example is also provided in table 2.2. The log transformation of the microarray data is commonly performed to allow easier association of the fold change with the "expression distance value" between two genes.

The functions of genes were deduced using the online tool PANTHER Classification System, supported by Applied Biosystems Inc., UK, (http://www.pantherdb.org/) (Thomas et al., 2003). The PANTHER database was also used to assign expressed genes to different categories based upon biological or molecular function (Mi et al., 2005;Mi et al., 2007). The number of genes in each category was compared to a reference list comprising all of the genes in the human genome using a binomial test available on the online database and applying the Bonferroni correction for multiple testing. By identifying over- and underrepresented classes of genes, an indication of processes activated or repressed within the oocytes and blastocysts was obtained.

For further analysis, the raw microarray data was entered into a Microsoft Office Excel 2007 spreadsheet and filtered, as before, for signal to noise ratios < 3 and flags over 5000. Lists of gene groups of interest were obtained from the PANTHER database, based on their Celera ID, and loaded onto a separate Excel spreadsheet. A Visual Basic for Applications language program was used to identify whether the genes of interest

appeared on the microarray expression list, and also whether their expression differed significantly between blastocysts and oocytes.

Table 2. 2: Logarithmic transformation of the microarray data. The log2 ratio facilitates the interpretation of fold change and difference in expression between two genes. The negative values indicate under-expression of a gene relative to a control.

	Expression ratio	**Log_2 of expression ratio**
Gene 1 (10 fold increase)	10	3.32
Gene 2	1	0
Gene 3 (10 fold decrease)	0.1	-3.32

3. Results

3.1 PGD for DM1

3.1.1 Protocol development

3.1.1.1 Patient details

All patients with DM1 referred for PGD, went through an initial consultation and provided blood samples for testing of genomic DNA, however, not all of them proceeded with the IVF/PGD treatment due to personal or medical reasons (Methods, section 2.1.1). The twenty-three patients and their partners who started IVF treatment with an aim to undergo PGD for DM1 are discussed here.

Out of the 23 patients, there were 19 affected females and 4 affected males. One patient was asymptomatic but the remaining 22 patients showed clinical signs of DM1. The most common reason for referral was the presence of a family history of DM1. Overall, 22 out of 23 patients had DM1-affected family members. Fourteen out of the 23 couples had not experienced a pregnancy, either because of their choice to avoid an affected pregnancy, or due to their inability to conceive naturally (seven couples). One couple had a natural unaffected pregnancy occurring after referral for PGD. The remaining eight patients with their partners had a personal experience of an affected pregnancy. In particular, two couples had lost a congenitally affected child, five had undergone termination of pregnancy (TOP) following Prenatal Diagnosis and one couple had an affected daughter and had also had two TOPs (figure 3.1, table 3.1). In the case of couple number 10, it was the birth of an affected child following the birth of two unaffected children that prompted the diagnosis of DM1 in the mother.

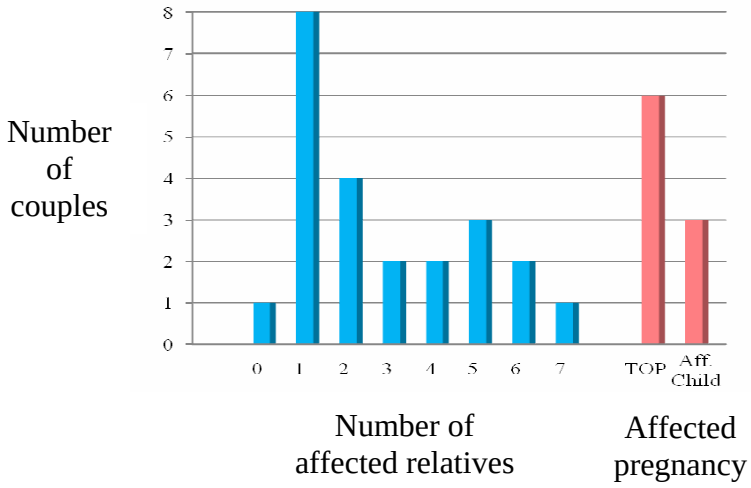

Figure 3. 1: Reasons to opt for PGD in our group of patients: Family History: 22 referred couples had one to seven affected relatives. Affected pregnancy: TOP: termination of pregnancy (six couples). Three couples had experienced one TOP, two couples had had two TOPs and another couple had three TOPs. One of the couples who had a TOP also had an affected daughter. Two other couples had a CDM1 baby.

Table 3. 1: DM1 patient history: The number of known affected relatives is shown for each patient and the number of these relatives who were first-degree is shown in brackets. Cases where an affected relative was an offspring are specified in the reproductive history column. Affected pregnancies are indicated in bold type. F: Female, M: Male, TOP, termination of pregnancy; CDM1, congenital myotonic dystrophy type 1; CVS, chorionic villus sampling; FSHD, facioscapulohumeral muscular dystrophy.

Patient number	Affected Partner	Total number of affected relatives (1st degree relatives)	Reproductive History
1	F	5 (3)	No previous pregnancies, infertile
2	F	1 (1)	Two **TOPs** following CVS
3	M	1 (1)	No previous pregnancies; partner infertile (low sperm count)
4	F	1 (0)	No previous pregnancies
5	F	1 (1)	One **TOP** following CVS
6	M	0	Four pregnancies, three **TOPs,** one unaffected child
7	F	3 (2)	One **TOP**; infertile (female anovulatory, partner has low sperm count)
8	F	2 (2)	No previous pregnancies
9	F	5 (2)	No previous pregnancies
10	F	1 (1)	Three pregnancies; two unaffected children (one from previous partner); **One CDM1 baby with current partner died**
11	F	4 (2)	No previous pregnancies
12	M	1 (1)	No previous pregnancies; partner infertile (azoospermia); used egg donor to avoid FSHD carried by partner
13	F	6 (2)	One **TOP** following CVS
14	F	5 (2)	No previous pregnancies; partner infertile (severe oligospermia)
15	F	2 (2)	**One CDM1 son** died at 18 hours; early miscarriage in second pregnancy
16	F	2 (2)	Natural unaffected pregnancy (occurred after referral for PGD)
17	M	2 (2)	No previous pregnancies
18	F	4 (4)	No previous pregnancies, infertile (has endometriosis and tubal disease)
19	F	1 (1)	No previous pregnancies
20	F	1 (1)	**2 TOPs** following PND, **affected daughter**
21	F	7 (1)	No previous pregnancies
22	F	6 (2)	No previous pregnancies
23	F	3 (2)	2 year history of subfertility (depleted ovarian reserve)

3.1.1.2 Genomic DNA PCR analysis

Genomic DNA from affected individuals and their partners was tested for the *DMPK* repeat expansion as well as polymorphic markers linked or unlinked to the *DMPK* gene. Figures 3.2 and 3.3 show examples of fluorescent PCR analysis for the main markers used (section 2.4.1, table A2.1).

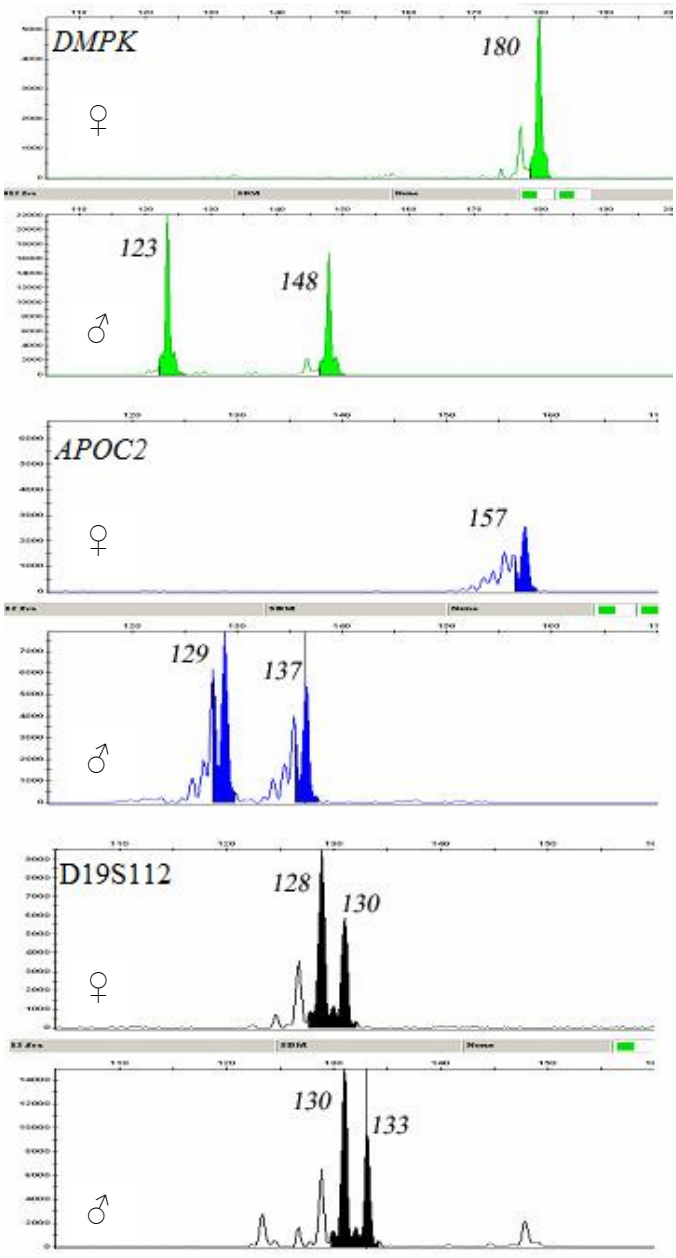

Figure 3. 2: ABI 3100 fluorescent PCR results for *DMPK*, *APOC2* and D19S112 polymorphic loci for couple number 19 (affected female). The x-axis shows the length of the PCR product in base pairs and the y-axis shows the fluorescence intensity in relative fluorescence units (RFU). The affected female shows only the non-expanded allele at the *DMPK* locus, as the allele with the expansion is refractory to PCR amplification. The markers are labelled with different fluorescent dyes to allow simultaneous analysis and can also be differentiated by their stutter pattern.Allele sizes are indicated in base pairs (bp) next to the corresponding peaks.

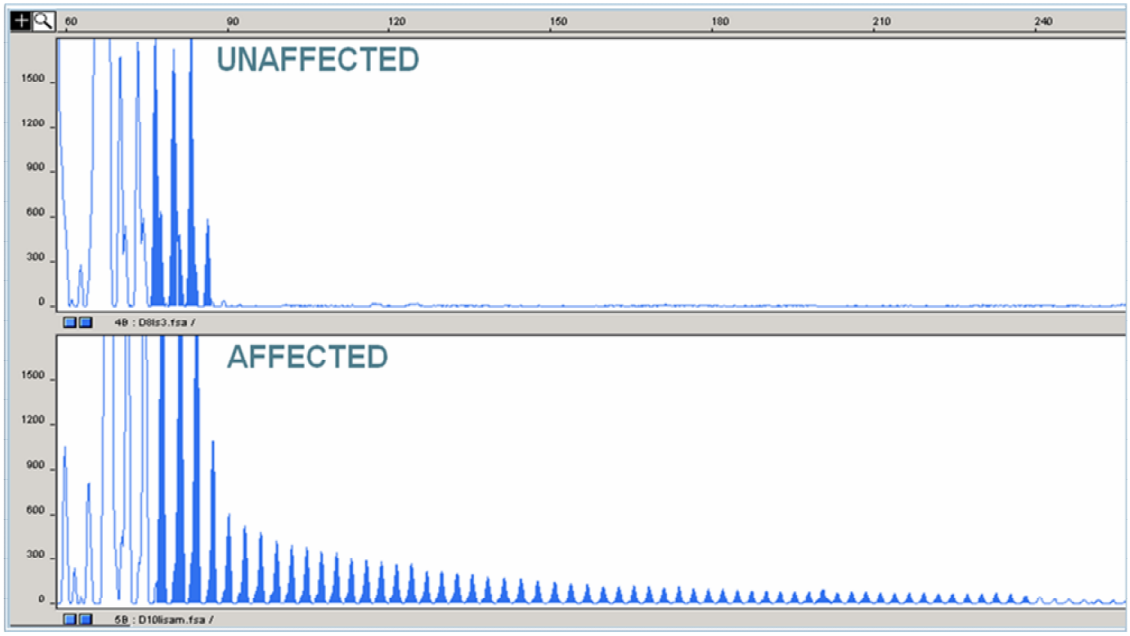

Figure 3. 3: Example of results from the TP-PCR protocol. Unaffected and affected individuals are differentiated by the number of peaks detected after amplification of the CTG repeat region. x-axis: PCR product size in bp, y-axis: fluorescence intensity (RFU).

Appendix table A2.1 shows the genetic analysis results for the *DMPK* mutated region as well as *APOC2* and D19S112 polymorphic markers for 23 couples (46 individuals) and 36 relatives. In an unaffected partner the two non-expanded alleles might be either of the same (homozygous) or of different (heterozygous) size. Examples of this are the unaffected males of couples 1 and 2 respectively (table A2.1). A summary of findings from marker analysis is given in tables 3.2 and 3.3 below. The F-PCR results for the other polymorphic markers tested are not shown here as they did not have clinical application.

Table 3. 2: Polymorphic marker analysis for 23 patients, their partners and relatives Thirty-seven out of the 82 individuals tested were unaffected (23 partners and 14 relatives). Heterozygosity for the triplet repeat region was calculated from the unaffected individuals only.

Marker	**Homozygous** (two same-size alleles)	**Heterozygous** (two different-size alleles)
CTG repeat		
Unaffected partners only	6/23 (26.1%)	17/23 (73.9%)
All unaffected partners and relatives	12/37 (32.4%)	25/37 (67.6%)
APOC2	13/82 (15.9%)	69/82 (84.1%)
D19S112	13/82 (15.9%)	69/82 (84.1%)

A couple was said to be informative, semi-informative or uninformative for a specific locus, based on the following definitions:

Informative: the couple have no alleles in common at a given locus. This allows clear differentiation of the maternal and paternal alleles in an embryo (e.g. couple no. 10 in appendix table A2.1 is informative for *APOC2* and D19S112 markers).

In several cases where a couple had no alleles in common at a specific locus, one or both of the partners were homozygous for the marker tested. In these cases, the couple was still considered to be informative as the maternal and paternal contribution would be identified in an embryo, however, detection of contamination would not be possible if testing only for that marker.

Semi-informative: the couple share one of their alleles at a given locus (e.g. couple number 1 is semi-informative for both *APOC2* and D19S112 markers).

Linkage analysis was performed for informative or semi-informative linked markers and, where the identified phase allele was not a shared allele, the use of these markers enabled indirect mutation detection.

Uninformative: the couple share all of their alleles at a given locus (e.g. couple no.12 is uninformative for *DMPK*).

Table 3.3 summarises the marker results regarding informativity for each of the couples, based on the information provided in the appendix table A2.1. Eleven out of 23 couples were informative for the *DMPK* repeat region, fifteen couples were informative for the *APOC2* polymorphic marker and thirteen couples were informative at the D19S112 locus. DNA from family members was available for 19 out of the 23 couples. In 2 cases CVS samples from previous pregnancies were available and in another two cases DNA was extracted from buccal cells of two affected children (couples no.5, 6, 10 and 20, appendix table A2.1). The phase of the linked markers, i.e. the allele that segregates along with the affected copy of the gene, can be detected by testing of affected or unaffected relatives, only for couples who are informative or semi-informative for the linked marker. The phase allele was not known for patients 1, 3, 11 and 23, as DNA from a relative was not available for the analysis. Similarly, the unaffected relative of patient 14 shared the same alleles for both markers as the patient, so the phase was not identified. For eight of the patients the phase alleles were identified at both marker loci, *APOC2* and D19S112, according to results on table A2.1.

Examples of the process of determining the phase are shown in figures 3.4 and 3.5. Apart from confirming the presence or absence of the mutation, polymorphic markers also allow detection of contamination and are therefore necessary in a PGD protocol in order to reduce the chance of misdiagnosis.

Table 3. 3: Marker informativity for the CTG repeat region, *APOC2* and D19S112 polymorphic markers Only one couple (no. 10) was informative for both the repeat region and the polymorphic markers. Overall, the phase allele for the D19S112 locus was known in 18/23 cases and this facilitated the diagnosis for those couples. hm: indicates cases where one or both of the partners were homozygous for the marker alleles.

Couple No.	Polymorphic marker			
	CTG repeat	***APOC2***	**D19S112**	**Phase known**
1	Informative	Semi-informative	Semi-informative	no
2	Semi-informative	Informative	Informative	yes
3	Informative	Informative (hm)	Semi-informative	no
4	Semi-informative	Informative	Informative	yes
5	Informative	Informative	Informative (hm)	yes
6	Informative	Informative	Semi-informative	yes
7	Uninformative	Informative	Semi-informative	yes
8	Uninformative	Informative	Semi-informative	yes
9	Uninformative	Semi-informative	Semi-informative	yes
10	Informative	Informative	Informative	yes
11	Informative	Informative (hm)	Semi-informative	no
12	Uninformative	Semi-informative	Informative	yes
13	Semi-informative	Semi-informative	Informative (hm)	yes
14	Uninformative	Informative	Informative (hm)	no
15	Semi-informative	Informative	Informative	yes
16	Semi-informative	Informative	Informative (hm)	yes
17	Informative	Uninformative (hm)	Semi-informative	yes
18	Informative	Uninformative (hm)	Informative	yes
19	Informative	Informative (hm)	Semi-informative	yes
20	*Uninformative*	Semi-informative	Informative	yes
21	Informative	Semi-informative	Informative	yes
22	Informative	Informative	Semi-informative	yes
23	Uninformative	Informative (hm)	Informative (hm)	no

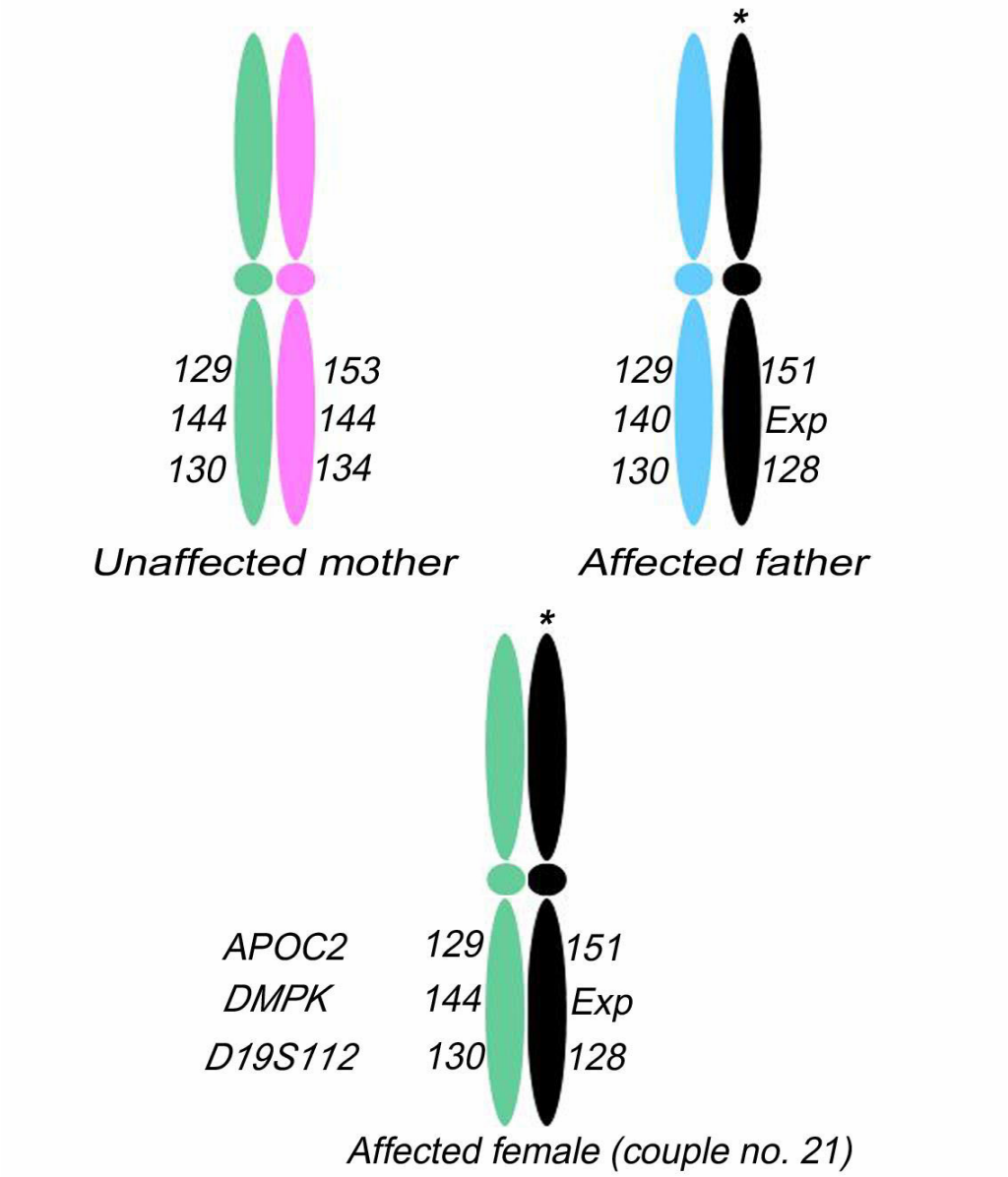

Figure 3. 4: Determining the phase for the affected female of couple no.21 The patient inherited the affected chromosome (*) from her father. Haplotype analysis shows that the affected female could only have got the 151bp and 128bp alleles from her affected father. Therefore, the *APOC2* 151bp marker allele and the D19S112 128bp allele seem to have segregated along with the affected copy of the gene. Detection of these alleles would confirm diagnosis of an affected embryo. It is advisable to test as many relatives and family generations as possible, in order to confirm the phase in a particular family.

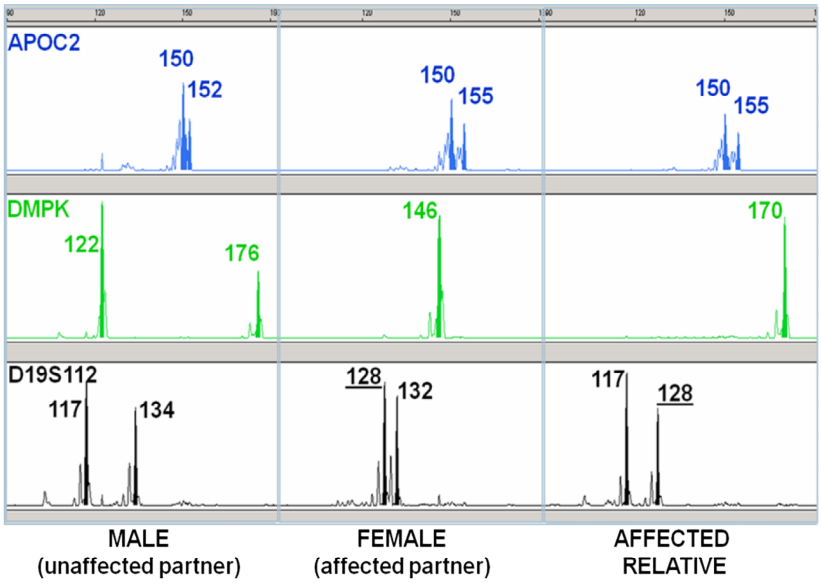

Figure 3. 5: Example of DM1 triplex PCR results for a couple and an affected relative (this couple chose not to undergo IVF treatment and is therefore not included in the list of patients in tables 3.1 or A2.1). x-axis: PCR product size in bp, y-axis: fluorescence intensity (RFU). Lane 1: *APOC2* locus, lane 2: *DMPK*, lane 3: D19S112. The affected female shows only the single non-expanded allele at the *DMPK* locus compared to her unaffected partner who has two normal-sized alleles of 122bp and 176bp size. The affected female and her affected relative share the 128bp allele at the D19S112 locus (underlined phase allele). The couple is semi-informative for *APOC2*. The affected female and her affected relative are uninformative for *APOC2* so the phase cannot be determined. The allele peaks are highlighted and the remaining peaks are stutters. Different stutter patterns are observed for each marker.

Based on the parental genotype and information on the phase alleles, all possible embryo genotypes can be predicted prior to the PGD case. Any deviation from the expected genotypes could indicate PCR contamination, allele dropout or cross-over events (figure 3.6).

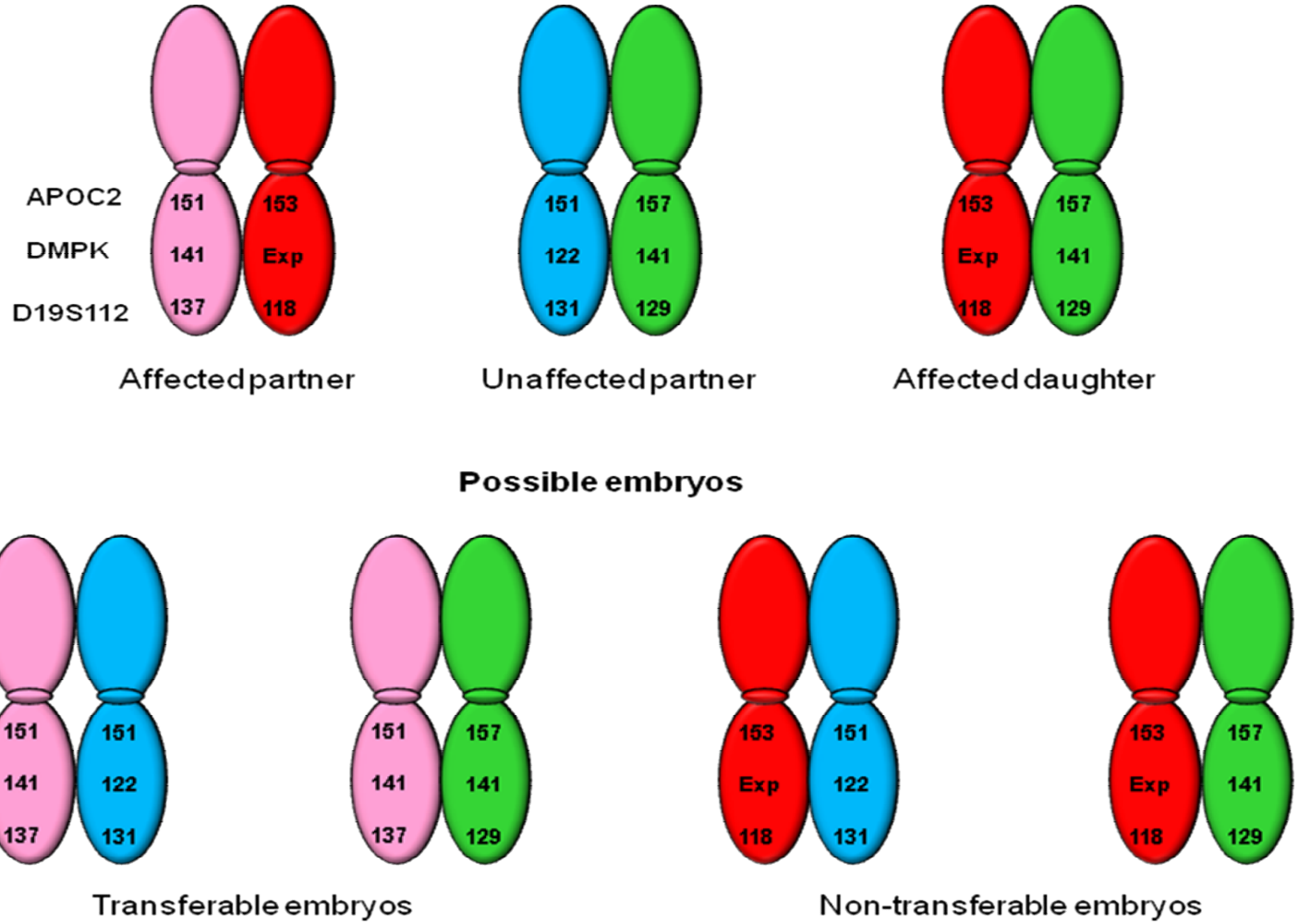

Figure 3. 6: Possible embryo genotypes for couple no. 20. The marker alleles on the affected copy of the gene were identified by PCR analysis of DNA from the affected daughter. Exp:expansion

3.1.1.3 Single cell optimisation

Each PGD protocol was optimised on single buccal cells or single lymphocytes using two different methods of lysis, either Proteinase K/SDS or alkaline lysis. The efficiency of diagnosis was determined by testing the final protocol on at least 50 cells prior to clinical application. The results from testing the protocol on single buccal cells or single lymphocytes for the five protocols with clinical application for the diagnosis of DM1 are shown in table 3.4 below (protocols 1-5) and details of the single-cell optimised PCR conditions are shown in table A2.2 of the appendix.

Optimisation involved modifications in the PCR program, concentration of reagents, as well as the incorporation of chemical additives, such as glycerol and DMSO in the PCR (Methods, section 2.4.1.1).

Differences were observed depending on the method of cell lysis and the type of cell used. A better amplification was achieved in protocol 2 (DM1/D19S112), when using ALB rather than PK/SDS lysis (testing on single buccal cells). In particular, DM1 and D19S112 amplification was 89.2% and 98.3% respectively with PK/SDS lysis *vs*. 96% and 100% respectively, when ALB lysis was used. The ADO rate following PK/SDS lysis was 28.97% for DM1 and 17.59% for D19S112, while following ALB lysis, the rate was reduced to 10% and 8% respectively. The drop in ADO at the *DMPK* locus with ALB vs. PK lysis was found to be statistically significant ($p<0.05$, Fisher's exact test).

In addition, amplification from single lymphocytes was higher and ADO rate was lower, compared to results from single buccal cells (table 3.4). The highest amplification (100%) was achieved for all loci of protocol 3 (DM1 triplex: DM1/*APOC2*/D19S112), following testing of single lymphocytes lysed with ALB. The ADO at the DM1 locus was 3.64%, lower than the ADO for DM1 when the same protocol was applied on single buccal cells (11.3%). The lowest ADO (0%) was achieved for the *APOC2* and D19S112 loci, contrary to the buccal cell results, which were 3.78% and 5.67% ADO respectively. TP-PCR amplification (protocol 4) was 99% from testing on single lymphocytes and 81.2% from single buccal cell analysis. This difference was also found to be statistically significant using the Fisher's exact test ($p<0.05$).

Protocols 3 (DM1/*APOC2*/D19S112) and 5 (TP-PCR/DM1/D19S112, described in more detail in the next section) involve multiplexing of more primer sets in a single PCR reaction compared to the other protocols. These new protocols minimised the work-up time for all patients with DM1 to less than one week. Following the initial

DNA extraction of parental or relative DNA, an appropriate protocol was selected depending upon the informativity of the couple.

Some protocols required results from two blastomeres of an embryo in order to achieve a diagnosis. A one-cell diagnosis could be obtained only for couples informative for the *DMPK* region and one or more of the linked markers, where the phase allele was also known.

A larger number of cells were tested with protocols number 4 (TP-PCR) and 5 (TP-PCR/DM1/D19S112) of table 3.4, due to several difficulties encountered with the TP-PCR protocol. In brief, suboptimal amplification and difficulties with sample analysis and scoring were observed, depending on the cell type and the size of the CTG repeat that was amplified. These are discussed further in sections 3.1.1.4 and 3.1.1.5. Results from two cells were always required for reaching a diagnosis when using any TP-PCR protocol.

Table 3. 4: Results from PCR analysis of single lymphocytes or single buccal cells for the five protocols with clinical application in PGD for DM1. Amplification and allele dropout rates were generally improved when using ALB rather than PK/SDS lysis, as well as when using single lymphocytes compared to single buccal cells. The protocols with the highest amplification efficiency and lowest allele dropout rates are highlighted in bold typing. *TP-PCR protocols are discussed in more detail in the following sections.

Protocol No. (markers)	**Cell lysis**	**Cell type**	**No. of Cells tested**	**Amplification (%)**				**Allele dropout (ADO) (%)**		
				P2/P3R/ P4CAG (TPPCR)	**DM1**	***APOC2***	**D19S112**	**DM1**	***APOC2***	**D19S112**
1 (DM1/*APOC2*)	PK/ SDS	buccals	69		68/69 (95.6%)	67/69 (97.1%)		7/68 (10.3%)	9/67 (13.4%)	
2 (DM1/ D19S112)	PK/ SDS	buccals	120		107/120 (89.2%)		118/120 (98.3%)	31/107 (28.97%)		19/108 (17.59%)
	ALB	buccals	50		48/50 (96%)		50/50 (100%)	5/50 (10%)		4/50 (8%)
3 (DM1/ *APOC2*/ D19S112)	ALB	buccals	53		53/53 (100%)	53/53 (100%)	53/53 (100%)	6/53 (11.3%)	2/53 (3.78%)	3/53 (5.67%)
	ALB	**lymphocytes**	**55**		**55/55 (100%)**	**55/55 (100%)**	**55/55 (100%)**	**2/55 (3.64%)**	**0/55 (0%)**	**0/55 (0%)**
4* (TP-PCR)	ALB	buccals	345	280/345 (81.2%)						
	ALB	lymphocytes	109	108/109 (99%)						
5* (TP-PCR/ DM1/ D19S112)	**ALB**	**lymphocytes**	**224**	**224/224 (100%)**	**224/224 (100%)**		**223/224 (99.6%)**	**1/89 (1.12%)**		**3/148 (2.03%)**

3.1.1.4 Standard PCR vs. TP-PCR

The techniques of standard PCR and TP-PCR were evaluated in terms of their ability to detect both normal-range and expanded CTG repeat alleles.

Standard PCR

With standard PCR amplification, the electropherogram of heterozygous unaffected individuals displayed two fluorescent peaks, representing the two non-expanded different-size alleles. Homozygous unaffected individuals (for example 12/12 CTG) showed one peak on analysis, as the amplification products from the two alleles were of equal size (in this example, two products of 143bp each). Although the expansion size of most affected individuals from our group of patients was not known, they were presumably all over 100 repeats, as no large alleles were detected by standard PCR. Therefore, for affected individuals (e.g. 12 CTG/expansion), where only the non-expanded allele could be amplified by standard PCR, electropherograms displayed only one peak (143bp) on analysis.

Standard PCR, therefore, cannot distinguish between homozygous unaffected samples from affected samples carrying the same size non-expanded allele.

TP-PCR

Amplification of unaffected heterozygous samples by TP-PCR produced a ladder, where the two main peaks, showing increased fluorescence signal, could be clearly visualized. Fluorescence intensity dropped to zero almost immediately beyond the larger allele peak. Contrary to standard PCR, TP-PCR could differentiate between the homozygous unaffected and affected genotypes by producing a characteristic amplification pattern from the expanded allele. The affected individuals, with over 100 CTG repeats present, gave a pathognomonic continuous ladder appearance following TP-PCR. An example of TP-PCR amplification of an affected sample was shown in figure 3.3. Figure 3.7 below gives a comparison of an affected *vs*. an unaffected sample,

carrying a large repeat allele and figure 3.8 indicates the TP-PCR ladder pattern for an individual carrying a 22 CTG repeat and a 50 CTG repeat allele.

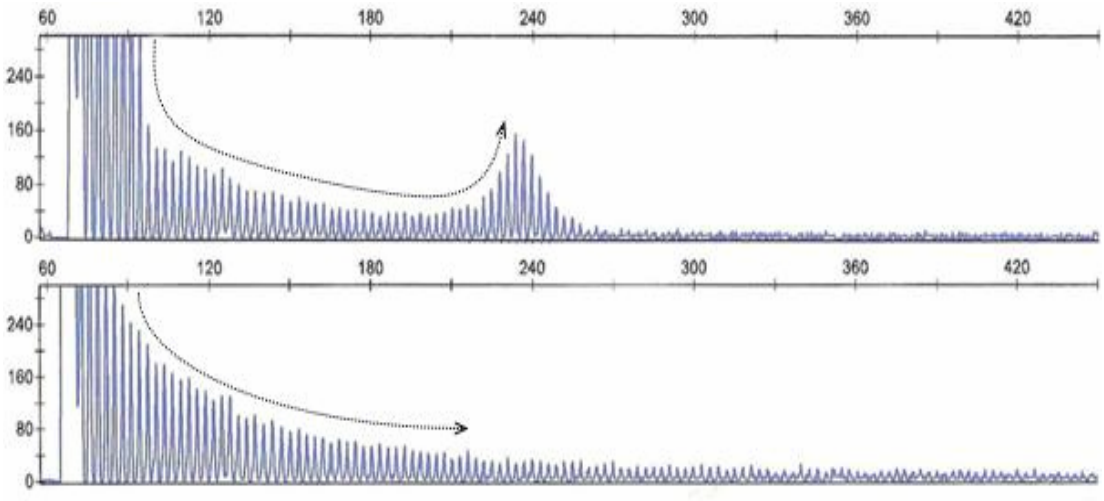

Figure 3. 7: TP-PCR amplification of a sample with a large DM1 allele and of an affected sample. x-axis: PCR product size in bp, y-axis: fluorescence intensity (RFU). In the first lane, the two DM1 alleles are indicated by the peaks of higher fluorescence, one near 95bp and the second one near 240bp. The fluorescence intensity reduces to zero shortly after the second high peak. In contrast, the affected sample, lane 2, shows the diminishing ladder pattern as in figure 3.3.

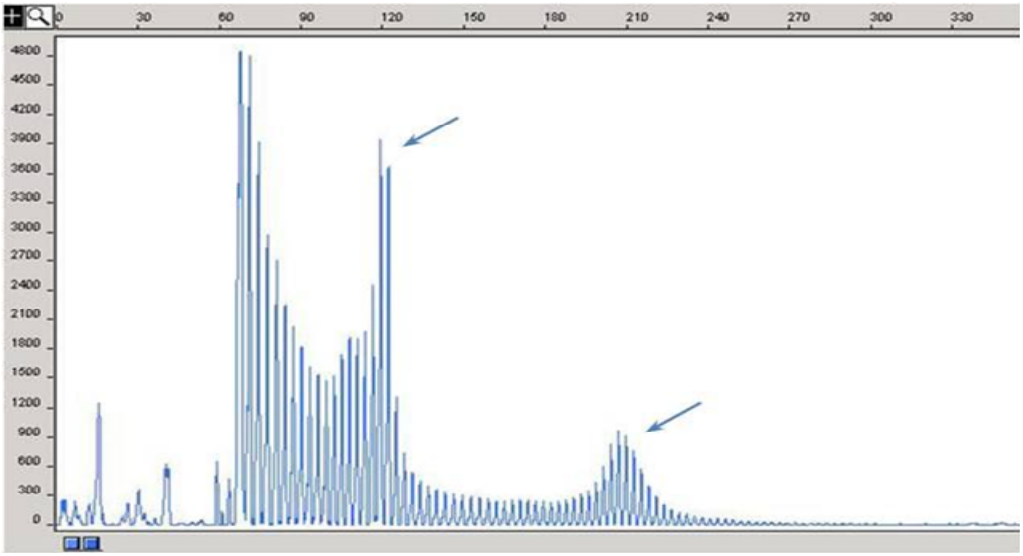

Figure 3. 8: TP-PCR amplification of a sample with 22/50 CTG repeat alleles. x-axis: PCR product size in bp, y-axis: fluorescence intensity (RFU). The two DM1 alleles are indicated by the peaks of higher fluorescence, one near 119bp and the second one near 207bp. The fluorescent intensity reduces to zero shortly after the second high peak.

The typical three-base pair ladder appearance of TP-PCR was not detected on analysis of the 5 CTG repeat homozygous samples. The presence of 5 repeats in the amplified region, corresponding to the 5 repeats of the P4CAG TP-PCR primer as in table A1.1, produced one peak, rather than a peak ladder, following amplification. This pattern could be mistaken for overall amplification failure (amplification of a degenerate or anucleate cell) or a case of ADO of a larger or maybe affected allele. The apparent lack of amplification in these cases was described as an inconclusive result, thus rendering TP-PCR diagnosis of 5 CTG repeat homozygous samples, problematic (figure 3.9).

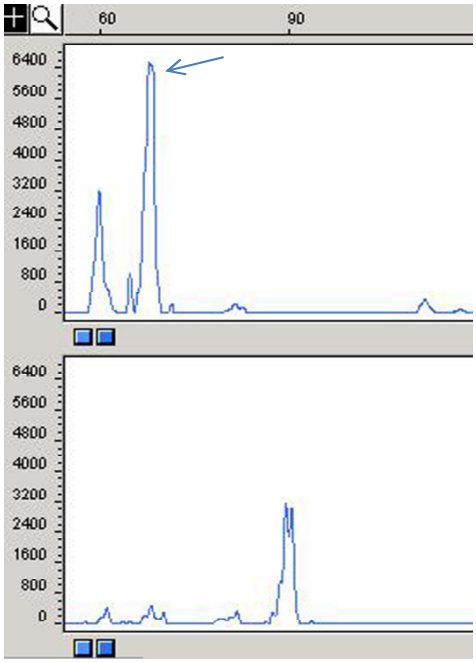

Figure 3. 9: TP-PCR of a 5 CTG repeat homozygous sample and a negative control. x-axis: PCR product size in bp, y-axis: fluorescence intensity (RFU). Top lane: the presence of the 5 CTG repeat is indicated by the 68bp amplification product. A triplet repeat ladder pattern is not detected. Bottom lane: the negative sample does not show amplification of the high 68bp peak. Random peaks, possibly primer-dimers, may appear in some negative controls. A triplet repeat ladder is not detected.

3.1.1.5 Multiplex TP-PCR/DM1/D19S112 PGD protocol (mTP-PCR)

Standard PCR and TP-PCR were combined in a single reaction in order to overcome the difficulties in diagnosis associated with each method, as described above. The standard PCR DMPK2 primer and the TP-PCR P2 primer were each labelled with a different fluorescent dye so that the resulting electropherograms allowed detection of the

genotype by both methods (figure 3.10). In this way, diagnosis of 5 CTG homozygous samples, where the ladder pattern was absent following TP-PCR giving the impression of failed amplification, was supported by the simultaneous evidence of a single amplified peak with the “standard PCR” amplification.

Because of the P2 and DMPK2 primers both being fluorescently labeled, the P2/DMPK2 product, as in figure 3.10, was seen in both the green and blue colours on analysis. Highly concentrated samples demonstrated additional bleed-through of the green into the blue fluorescent dye during fluorescent analysis. Dilution of the PCR product overcame this problem. Examples of results are shown in figure 3.11.

Following initial testing of the DM1/TP-PCR protocol on genomic DNA and single cells, the D19S112 linked marker was also incorporated in the reaction. This new protocol (mTP-PCR) provided additional information on contamination, ADO as well as enabling detection of the “phase allele”, where possible (figure 3.12).

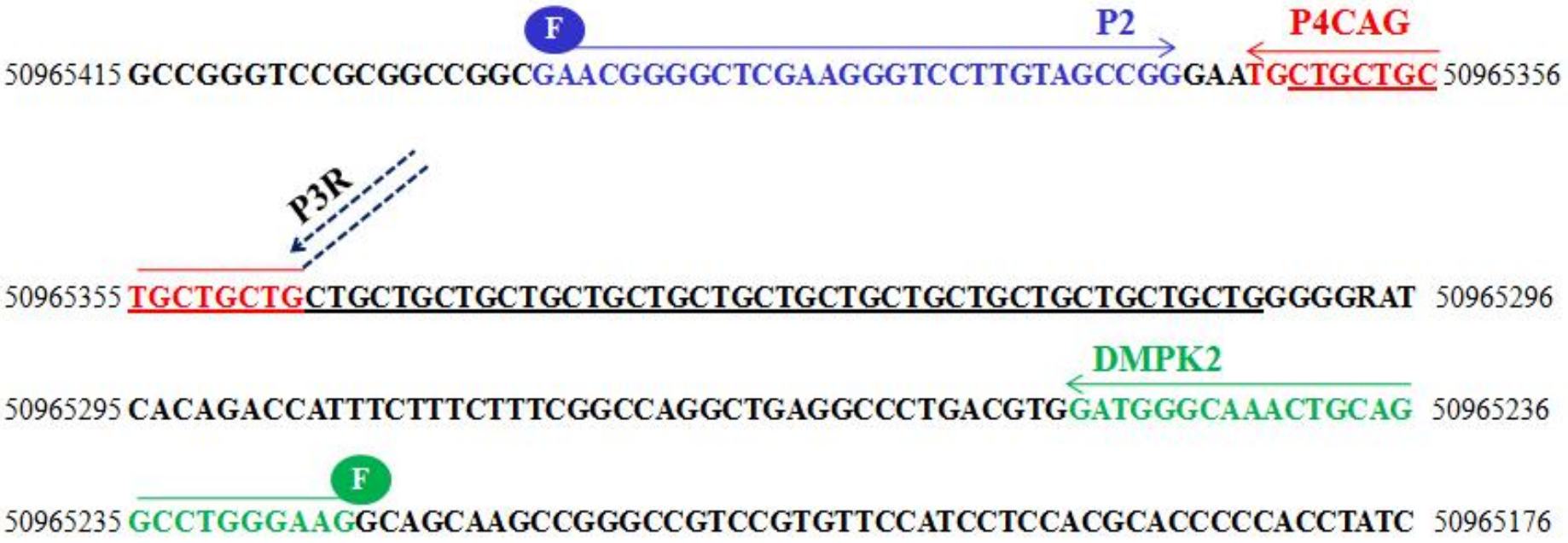

Figure 3.10: TP-PCR/standard PCR multiplex protocol design showing location of primers relevant to the triplet repeat region (underlined sequence). This protocol allows simultaneous TP-PCR amplification (primers P2, P4CAG, P3R) and standard PCR amplification of non-expanded alleles (P2/DMPK2 primers).

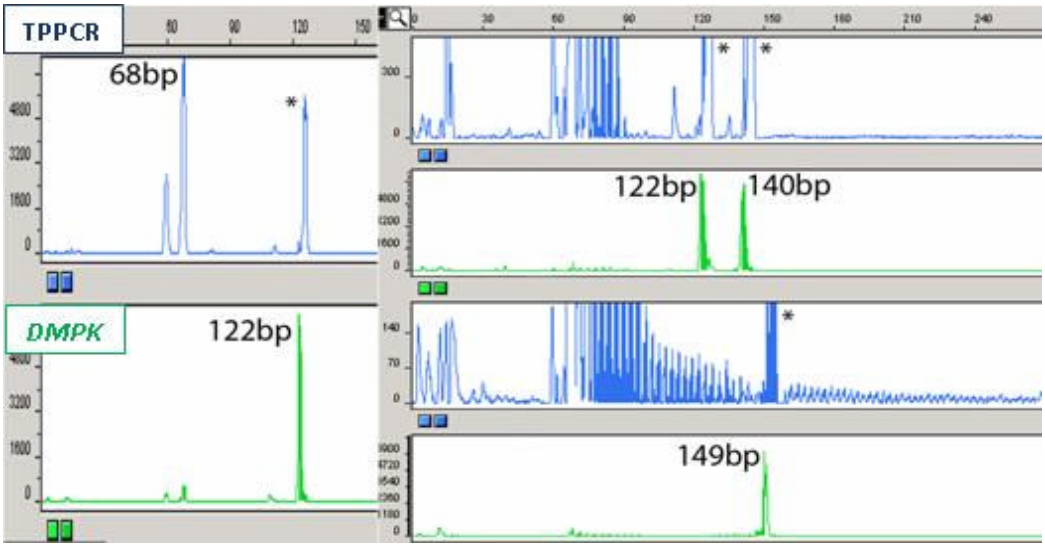

Figure 3.11: Results of TP-PCR and DM1 multiplex amplification. TP-PCR P2 primer is labelled in FAM fluorescent dye (blue colour) and DMPK2 primer is labelled in VIC (green colour). x-axis: PCR product size in bp, y-axis: fluorescence intensity (RFU). The 5 CTG repeat homozygous sample on the left side, indicates the presence of a 68bp peak with no triplet repeat ladder and a 122bp product with standard PCR amplification. A range of peaks is detected at TP-PCR for the 122/140bp sample (right side, top) while a diminishing ladder pattern is detected for the 149bp/expansion affected sample. * indicate the P2/DMPK2 standard PCR peaks that are detected in both the green and the blue fluorescent dye.

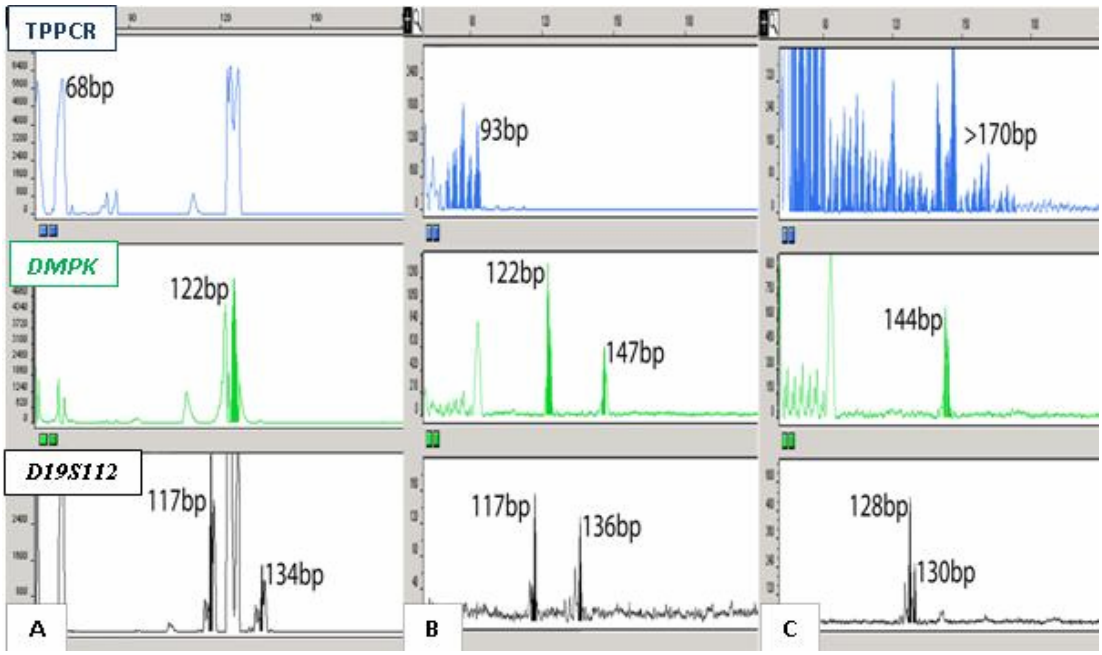

Figure 3.12: mTP-PCR results: A) 5 CTG repeat homozygous unaffected individual B) heterozygous unaffected individual C) Affected individual. x-axis: PCR product size in bp, y-axis: fluorescence intensity (RFU). The size of the last sizeable peak is indicated in base pairs (bp).

3.1.1.6 Comparison of mTP-PCR with TP-PCR amplification

Amplification with standard TP-PCR and mTP-PCR was compared by the parallel PCR and analysis of several genomic DNA samples (table 3.5). A similar pattern of amplification was detected between the two protocols; therefore, the inclusion of the DM1 and D19S112 markers in the mTP-PCR protocol did not seem to influence the amplification. For five individuals the size of the DM1 expansion was known, as indicated in table 3.5. The size of the last ladder peak, as detected by the genetic analyzer, did not correlate with the expansion size. This observation was made following amplification for both protocols.

Table 3. 5: Comparison of TP-PCR and mTP-PCR amplification of 16 genomic DNA samples. The size of the largest sizeable peak at the 310 genetic analyzer is indicated. The size of the last peak of the TP-PCR ladder that could be seen but not sized by the machine is given in brackets.

Case Number/ DNA source (number of repeats)	TP-PCR	mTP-PCR
4/ Affected female (580)	169 (174)	165 (171)
8/ Affected female	153 (265)	134 (206)
9/Affected female	162 (392)	177 (279)
9/ Female's father	246 (263)	302 (317)
10/ Affected female	127 (246)	230 (377)
10/Affected son (<1000)	186 (306)	183 (275)
13/ Affected female	258 (398)	259 (313)
17/ Affected male (108)	168 (311)	180 (290)
17/ Male's brother (200)	186 (339)	213 (358)
19/ Affected female	134 (191)	126 (130)
19/ Female's father	127 (251)	210 (250)
20/ Affected female	180 (351)	171 (293)
21/ Affected female	174 (311)	198 (311)
21/ Female's father	162 (419)	198 (449)
22/ Affected female	155 (251)	173 (298)
23/ Affected female (250)	195 (389)	177 (310)

3.1.1.7 Summary of TP-PCR and mTP-PCR protocol optimisation

Figure 3.13 summarizes the steps towards TP-PCR optimisation and development of the mTP-PCR protocol.

Scoring of unaffected samples following TP-PCR amplification was performed on an individual-specific basis. A minimum of twenty single cells isolated from each individual were tested using TP-PCR, to determine the size of the last ladder peak following amplification, indicating the size of the larger non-expanded allele. Following the testing on single cells, blind experiments were performed, where cells from a known unaffected individual were isolated along with cells from affected individuals. The tubes were randomly labelled by a laboratory colleague so that the results could be scored blindly following amplification. During blind scoring, any samples showing even a slightly larger than the expected product size, based on results from prior optimisation using the known unaffected individual's cells, were scored as affected. This was because it was thought that they could indicate suboptimal amplification of an affected sample, although they could also be due to polymerase slippage or the true presence of a slightly larger, not in the affected range, repeat.

These false-positive (FP) results, i.e. the number of unaffected cells scored as affected over the total number of unaffected cells tested, as indicated in figure 3.13, therefore, include samples where although the TP-PCR ladder did not show the characteristic diminishing pattern of an affected sample, the last sizeable peak was larger than expected. The number of these FP results is, therefore, thought to be possibly inaccurate, especially since TP-PCR scoring generally improved with experience. It is worth mentioning however, that even at the early stage of TP-PCR testing, buccal cell amplification with PK lysis produced overall inconsistent results and presented difficulties with scoring.

Additionally, more FP results were obtained with blind testing of buccal cell amplification following ALB lysis rather than lymphocytes following ALB lysis.

The uncertainties with scoring were avoided during a PGD case, as corresponding results from two cells were required for a diagnosis from each embryo.

The false negative (FN) results indicate the number of affected cells scored as unaffected over the total number of affected cells. FN results were only observed with buccal cell testing. Multiplex PCR protocols allowing TP-PCR amplification along with one or two polymorphic markers (initially TP-PCR/DM1 or TP-PCR/D19S112, followed by mTP-PCR), reduced the number of both FP and FN results to zero.

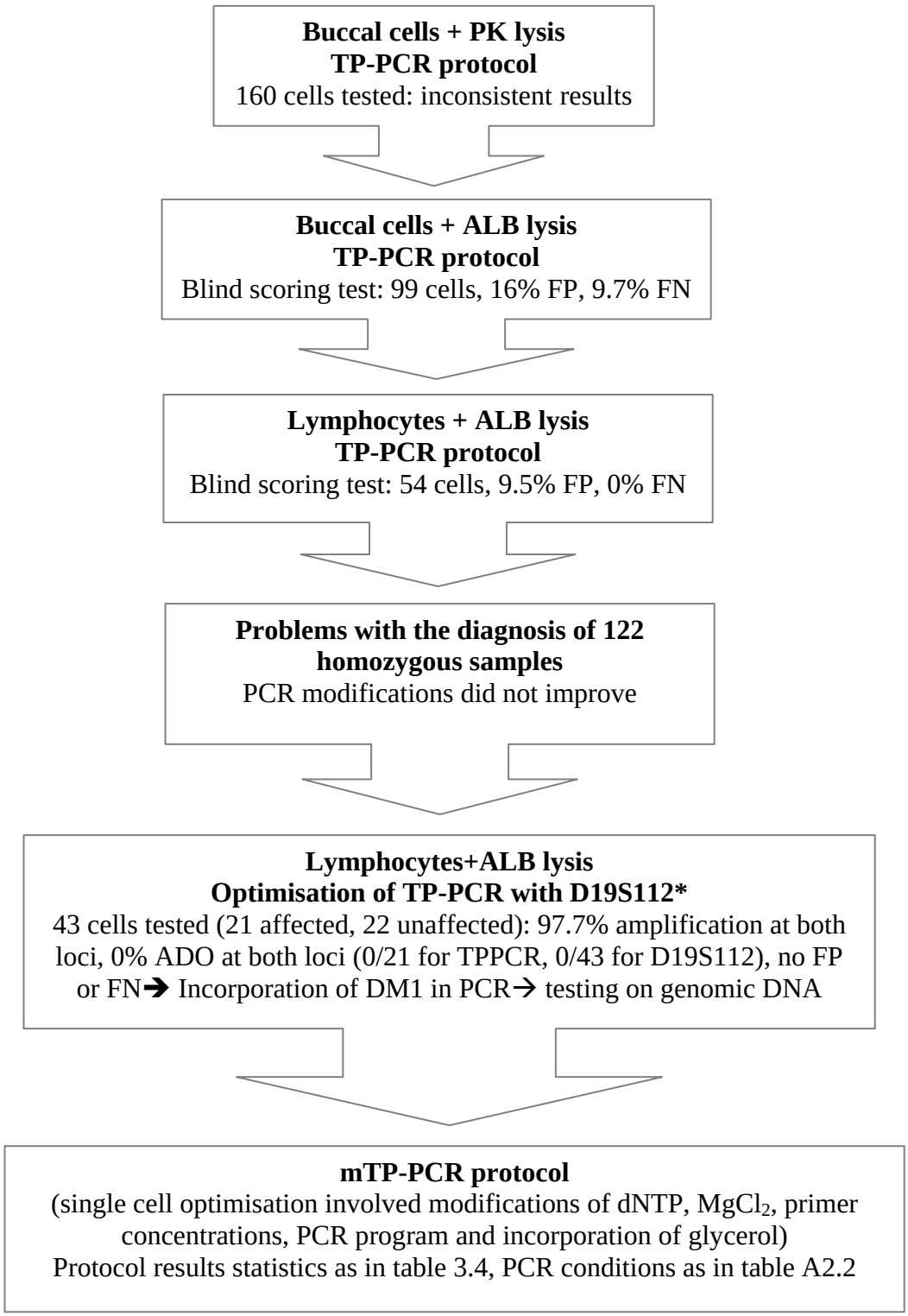

Figure 3. 13: Development of the mTP-PCR protocol. Blind scoring tests were performed during initial testing of the TP-PCR protocol. Scoring improved with the use of single lymphocytes compared to buccal cells and with experience. Table 3.4 indicates amplification results when using the optimised TP-PCR and mTP-PCR protocols. * Protocol TP-PCR/D19S112 is not included in table 3.4 as it did not have clinical application. Amplification results are indicated here. Details of PCR setup and PCR program for all protocols are indicated in the appendix table A2.2. FP: false positive, FN: false negative.

3.1.1.8 mTP- PCR and large non-expanded DM1 allele

Patient number 19 had the largest non-expanded DM1 allele from our group of patients, with 24 CTG repeats. Diagnosis for this patient was performed using the DM1 triplex protocol, as the couple was informative for DM1 and *APOC2* loci, semi-informative for the D19S112 locus and the phase allele was known. In order to test amplification of large DM1 alleles with the mTP-PCR protocol, cumulus cells were collected following egg collection during the patient's IVF/PGD cycle. Amplification of single cumulus cells as well as of clumps of cumulus cells, showed lower amplification for the large DM1 allele (approximately 100 fluorescent units), when compared to the TP-PCR ladder and the D19S112 marker result (figure 3.14).

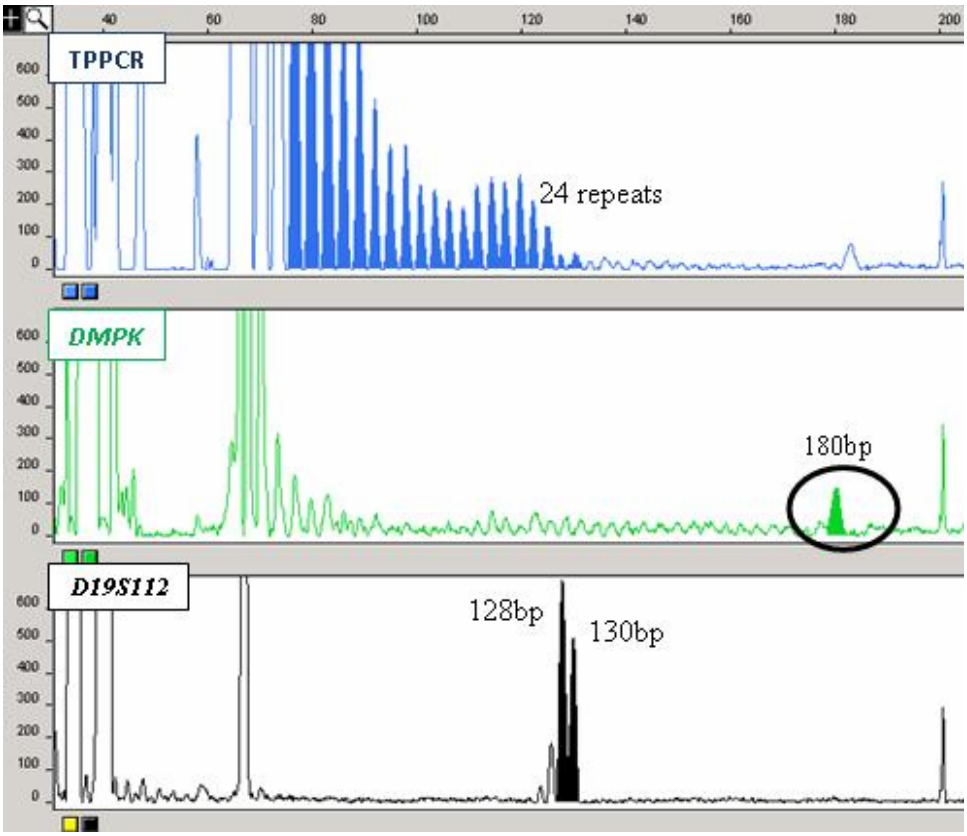

Figure 3.14: mTP-PCR amplification of a cumulus cell clump of patient 19. x-axis: PCR product size in bp, y-axis: fluorescence intensity (RFU). Amplification of the DM1 180bp allele (24 repeats, as sized by TP-PCR) was compatively low, prompting further investigation by testing of single lymphocytes.

Twenty single lymphocytes were also isolated and tested with the mTP-PCR protocol from this patient. Analysis confirmed general failure of amplification for the large non-expanded DM1 allele compared to results from amplification of single lymphocytes

from other DM1 patients, where the size of the non-expanded DM1 allele ranged from 122bp-149bp (table 3.6). Prior to this observation protocol optimisation had focused on single cell amplification from these other patients, as they were the ones in need of an improved PGD protocol because of their uninformativity for DM1 and/or linked markers. Several cells had also been tested from patient number 22 (affected), who had a 168bp DM1 allele, but problems with amplification were not observed in this case.

Table 3. 6: mTP-PCR lymphocyte amplification for different sized non-expanded DM1 alleles. Low amplification was detected for the larger 180bp DM1 allele of patient 19

Non-expanded DM1 allele of affected individual	**Cell type**	**No. of cells tested**	**Amplification (%)**			**Allele dropout (ADO) (%)**	
			P2/P3R/ P4CAG (TP-PCR)	**DM1**	**D19S112**	**DM1**	**D19S112**
122-168bp DM1 alleles	lymphocytes	224	224/224 (100%)	224/224 (100%)	223/224 (99.6%)	1/89 (1.12%)	3/148 (2.03%)
180bp DM1 allele (patient 19)	lymphocytes	20	20/20 (100%)	7/20 (35%)	20/20 (100%)	n/a	0/20

Further investigation of amplification of the larger DM1 allele was performed by testing embryos from this couple that were donated for research following PGD. In particular, three unaffected 2PN embryos and one unaffected 0PN were re-tested for this investigation. Spare embryos were disaggregated and single blastomeres were tubed in either DTT/NaOH or DTT/KOH, to allow comparison of amplification from blastomeres of the same embryo using either the DM1 triplex protocol (DM1/*APOC2*/D19S112) or the mTP-PCR protocol, which required different cell lysis conditions (table 3.7). Several blastomeres were also saved for MDA amplification. These are further discussed in section 3.2.1.3.

In summary, four blastomeres were tested with the DM1 triplex and all showed successful amplification with no allele dropout for all markers. Six blastomeres were tested with the mTP-PCR protocol and all showed amplification at all loci. No allele dropout was detected for D19S112, while all cells showed ADO of the large 180bp DM1. This ADO would not hinder successful diagnosis in any of the cases, due to the

presence of the D19S112 marker and the absence of the phase allele on analysis. Amplification from some of the single blastomeres indicated a product over 125bp in size, which would be the expected for amplification of 24 repeats.

Unfortunately, following this PGD cycle, there were no more cells from a patient with a similarly large normal DM1 allele available for further testing and optimisation.

Table 3. 7: Testing of blastomeres from four embryos, diagnosed as unaffected during PGD for patient 19. ADO of the 180bp DM1 allele was observed with the mTP-PCR protocol. The TP-PCR column indicates the size of the last sizeable peak at the 310 genetic analyzer. Exp: expansion

	Cell number	**Protocol number**	**F-PCR reading**			
Parental genotype			**TP-PCR**	**DM1**	**D19S112**	***APOC2***
Affected female			186	180/Exp	128/130	157/157
Unaffected male			100	122/148	130/133	129/137
Unaffected Embryo tested/ PN scoring						
1/2PN	1	DM1 triplex		122/180	128/130	129/157
	2	mTP-PCR	126	122/**ADO**	128/130	
	3	mTP-PCR	136	122/**ADO**	128/130	
2/2PN	1	DM1 triplex		148/180	128/133	137/157
	2	mTP-PCR	131	148/**ADO**	128/133	
	3	mTP-PCR	131	148/**ADO**	128/133	
3/2PN	1	DM1 triplex		148/180	128/133	137/157
	2	mTP-PCR	141	148/**ADO**	128/133	
4/0PN	1	DM1 triplex		122/180	128/130	129/157
	2	mTP-PCR	134	122/**ADO**	128/130	

3.1.2 Results and follow-up from DM1 PGD cycles

3.1.2.1 Response to IVF treatment and embryo biopsy

Five out of the 35 IVF/PGD treatment cycles started were cancelled before oocyte retrieval due to either poor response to the IVF treatment (patient number 5, 14, 17 and 18) or hyperstimulation (patient number 4, first cycle). In another two cycles oocytes were collected and fertilized but there were only two embryos available for biopsy on day 3. In both cases PGD was cancelled and the embryos were discarded.
From the remaining 28 cycles, 317 oocytes were collected, 285 of which were mature and were inseminated and 177 were normally fertilised (2PN) (62.1% fertilisation rate). The average maternal age was 33.4±3.1 years. Seventy-four oocytes were abnormally fertilized (sixty-six 0PN, six 1PN and two 3PN), three oocytes cleaved prematurely and one oocyte was described by embryologists as being “out of the zona membrane”. The remaining thirty oocytes had disintegrated following insemination (table 3.8).

One hundred and sixty seven out of the 177 (94.4%) of the 2PNs were of sufficient quality for biopsy on day 3 post fertilisation. Five 0PNs, one 1PN and one 3PN from four cycles, had grown to the 6-8 cell stage and were also biopsied (table 3.9). The total number of biopsied embryos was, therefore, 174.

Table 3. 8: Summary of oocytes collected and inseminated by ICSI for DM1 PGD cycles. The seven cancelled cycles are marked in red colour. Normally fertilized oocytes showed two pronuclei (2PN) 18-20 hours post-insemination. 0PNs, 1PNs, 3PNs and other abnormalities are also indicated. Thirty oocytes disintegrated post-insemination. 'Total' numbers shown do not include the cancelled cycles. Yrs: years. Disint: disintegrated, PC: prematurely cleaved, Z: out of zona. *2PN from a giant ovum

Patient Number/ Cycle	Female Age (yrs)	Oocytes collected	Oocytes inseminated	2PN	0PN	1PN	3PN	Disint.	Other
1/1	33	7	7	3	4				
2/1	34	10	5	4	1				
3/1	*28*	*7*	*7*	*2*					
4/1	*27*	*Hyperstimulation*							
4/2	27	11	10	7	3			2	
5/1	*29*	*Poor response*							
6/1	36	13	13	11					
7/1	35	18	16	10	6				
8/1	33	9	9	6	3				
8/2	34	16	14	5	4			4	1PC
8/3	35	13	12	3*	7			2	
9/1	27	6	5	4	1				
9/2	28	13	10	8					
9/3	*29*	*7*	*6*	*2*					
10/1	34	9	9	5	2	1		1	
11/1	33	14	14	12	1				1Z
11/2	34	19	17	15				2	
12/1	35	12	11	3	8			2	
13/1	33	13	12	4	3	3			2PC
13/2	33	20	20	10	2	1		7	
13/3	34	15	13	4	6	1			
14/1	*36*	*Poor response*							
15/1	39	9	8	6	2				
16/1	34	10	10	10					
17/1	*35*	*Poor response*							
18/1	*37*	*Poor response*							
19/1	30	11	11	6	3			2	
19/2	31	12	10	6	2			2	
19/3	31	9	8	5	3				
20/1	35	6	4	2	1		1		
21/1	31	9	9	8				1	
22/1	34	16	13	9	2			2	
22/2	35	8	6	5	1				
23/1	39	4	4	3			1		
23/2	39	5	5	3	1			1	
Total	33.4±3.1	317	285	177	66	6	2	30	4

Table 3. 9: Details of embryos biopsied from each of 28 PGD cycles. PN: pronuclei**,** 2PN: two pronuclei seen, 0PN: no pronuclei seen

Patient number/ Cycle number	Embryos biopsied		
	2PN	0PN	Other
1/1	3	1	
2/1	4		
3/1	n/a		
4/1	n/a		
4/2	7		
5/2	n/a		
6/1	10		
7/1	10		
8/1	6		
8/2	5		
8/3	3		
9/1	4		
9/2	8		
9/3	n/a		
10/1	4		
11/1	12		
11/2	15		
12/1	3		
13/1	4		
13/2	10		
13/3	4	3	1 (1PN)
14/1	n/a		
15/1	6		
16/1	10		
17/1	n/a		
18/1	n/a		
19/1	2		
19/2	5		
19/3	5		
20/1	2	1	1 (3PN)
21/1	7		
22/1	7		
22/2	5		
23/1	3		
23/2	3		
Total	167	5	2

Most embryos (119/174, 68.39%) had two cells biopsied. On several occasions, however, it was necessary to remove more than two cells from a single embryo (figure 3.15).

If during biopsy on day 3 a biopsied cell was found to be anucleate or lysed, then an additional cell was removed if the embryo development stage permitted (>6 cells remaining, see methods section 2.1.3). In some cases additional cells were removed on day 4, if results from previously biopsied cells on day 3 had not yielded a clear diagnosis (detection of cells showing amplification from one parental genome, cross-over, allele dropout, contamination or amplification failure) or if the embryo stage on day 3 had not permitted biopsy of two cells at that time.

In cases where the protocol required results from two cells if, on day 3, the embryo development stage only permitted the removal of one cell, a re-biopsy was performed on day 4 to obtain confirmation of diagnosis for embryos with an unaffected result.

Re-biopsy was performed in 6 PGD cycles, for a total of 16 embryos. In four of the 6 cycles this was for confirmation of an unaffected embryo in a two-cell requiring protocol.

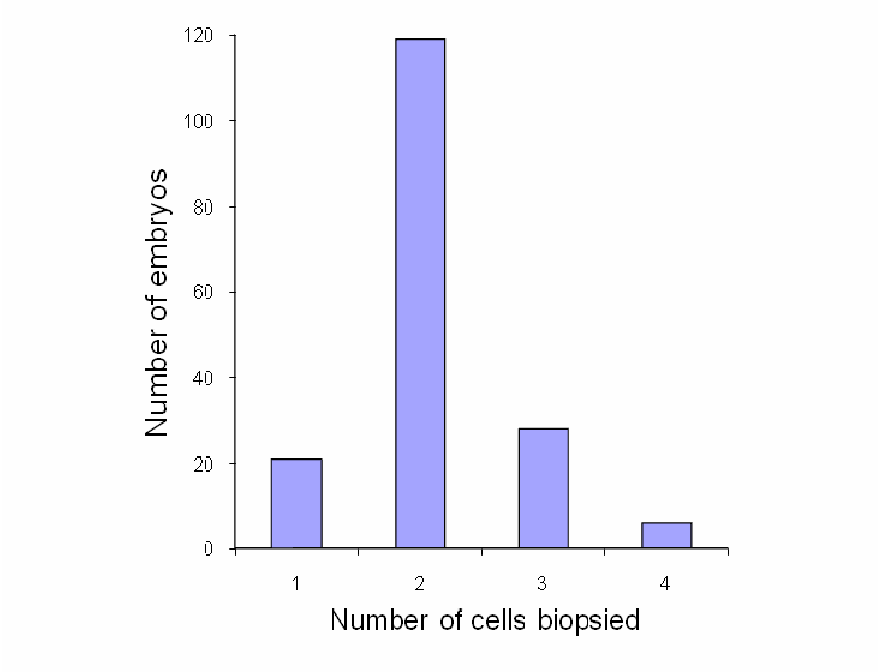

Figure 3. 15: Number of embryos with one to four cells biopsied. Graph includes the sixteen rebiopsied embryos

Amplification rate, allele dropout and overall diagnosis rate per protocol used are summarised in table 3.10. A more detailed analysis of blastomere amplification for each case and protocol used may be found in the appendix tables A2.3-A2.7. For one couple, the diagnosis was performed using two different protocols.

Table 3. 10: Summary of blastomere results from all DM1 PGD cases showing overall amplification, allele dropout and diagnosis rate per protocol. The allele dropout for the DM1 locus could be obtained only from the unaffected heterozygous cells. A more detailed analysis per case is shown in table A2.3-A2.7 of the appendix. Protocol 1a is a modification of protocol 1, involving a 'split PCR' reaction, which was performed in one PGD cycle, as indicated in the appendix. The best protocols in terms of amplification, ADO rate and number of markers tested, are indicated in bold typing. H: embryos where the two biopsied cells indicated amplification from only one of the parental genomes *: diagnosis for cycle 2 of patient no.8 was performed using two different protocols, thus making the total number of cases reported here 29.

Protocol Number (markers)	**No. of cases***	**No. of embryos**	**No. of blastomeres**	**Amplification**				**Allele dropout**			**Diagnosis**	
				P2/P3R/ P4CAG	**DM1**	***APOC2***	**D19S112**	**DM1**	***APOC2***	**D19S112**		
1 (DM1/ *APOC2*)	4	30	48		38/48 (79.2%)	35/48 (72.9%)		1/12 (8.3%)	3/29 (10.3%)		19/30 (63.33%)	
1a	1	4	6		5/6 (83.3%)	3/6 (50%)		n/a	1/3 (33.3%)		3/4 (75%)	
2 (DM1/ D19S112)	2	11	24		21/24 (87.5%)		20/24 (83.3%)	0/3		1/13 (7.7%)	8/11 (72.7%)	
3 (DM1/ *APOC2*/ D19S112)	**11**	**69**	**122**		**110/122 (90.2%)**	**107/122 (87.7%)**	**107/122 (87.7%)**	**0/43**	**0/80**	**0/70**	**52/69 (75.36%)**	**4H (non-transferable) 56/69 (81.2%)**
4 (TP-PCR)	5	37	75	46/56 (82.1%)							30/37 (81.08%)	
5 (mTP-PCR)	**6**	**28**	**60**	**52/60 (86.7%)**	**48/56 (85.7%)**		**52/60 (86.7%)**	**1/6 (16.7%)**		**0/40**	**22/28 (78.57%)**	**4H (non-transferable) 26/28 (92.9%)**

All cases where amplification from only one of the parental genomes was detected, are indicated in the appendix 2 tables A2.3, A2.5 and A2.7. When both of the biopsied cells indicated alleles from one of the parents only and considering a low chance of ADO simultaneously occurring at all three amplified loci, the embryo was scored as potentially indicating monosomy for chromosome 19.

Five multinucleate blastomeres were biopsied in three cycles from patients/cycle number 6/1, 11/1 and 12/1 (patient 12 had two multinucleate cells biopsied from one embryo and another multinucleate cell from a different embryo). Additionally, four binucleate blastomeres were biopsied from patients/cycle number 13/1, 13/2, 19/3 and 22/2, and in another two cases, for patients/cycle number 11/2 and 21/1, micronuclei were observed during biopsy in all embryos and in one of the biopsied embryos respectively.

Three out of the five multinucleate cells gave an unaffected result on diagnosis, and the two cells that were biopsied from the same embryo (patient/cycle 12/1) gave an inconclusive result (paternal contribution on one marker, shared alleles on the other two markers). All four embryos, corresponding to these biopsied multinucleate cells, were of poor quality. Two of them had arrested at the four-cell stage, as scored on day 4 and 5, and the other two embryos had seven and eight cells on day 4. One of these embryos, the 8-cell embryo of patient/cycle 12/1, was transferred but a pregnancy was not established.

Of the binucleate cells, three gave a result on analysis, two of them, patient/cycle 13/2 and 19/3, indicated affected embryos and the third one, patient/cycle 22/2, indicated amplification of the maternal genome only (affected). Scoring of these three embryos on day 4 indicated two 6-cell embryos (patients 13 and 22) and a 11-cell embryo (patient 19). The binucleate cell that failed to give a result (patient/cycle 13/1) came from an embryo where other biopsied cells gave an unaffected result on diagnosis. This embryo had eight cells on day 4 and was transferred but a pregnancy was not established. One of the cells where micronuclei were observed did not give a result. In the other case (11/2) where micronuclei were generally observed during embryo biopsy, there were no problems with diagnosis; two embryos were transferred on day 5, both blastocysts, both of which implanted.

Thirty-three cells began to lyse during the embryo biopsy and tubing procedure, however, the nucleus was seen in all cases by the embryologists and the cells were tubed without delay. Only four out of these 33 cells failed to amplify. On the contrary,

forty-two cells were considered to be cytoplasmic fragments or anucleate cells during the biopsy and all, apart from one, failed to give a result following PCR. Results from analysis of all cells recorded as fragments and anucleate cells during biopsy were excluded from the investigation of protocol efficiency and diagnosis rate of table 3.10.

Protocols DM1/APOC2/D19S112 and mTP-PCR provided the most information from a single cell. These protocols allowed the identification of eight embryos where, in both cells, only one of the parental genomes was detected. It is likely that these embryos are true haploid embryos or embryos with monosomy 19, as the results were confirmed from the amplification of three markers in two cells from each embryo. A diagnosis rate of 75.36% and 78.57% was achieved with these two protocols respectively (table 3.10 also indicates the potential diagnosis rate of 81.2% and 92.9%, to include the eight embryos where the presence of only one parental genome was detected).

Protocols DM1/*APOC2* (1, 1a) and DM1/D19S112 were associated with low amplification and diagnosis rate (63.33%, 75% and 72.7% respectively). The TP-PCR protocol, which is commonly used for DM1 testing, gave a diagnosis of 81.08%, however this protocol requires results from two cells from an embryo for diagnosis, while providing no information regarding the presence of contamination.

Overall, from all 28 DM1 PGD cases completed, using all of the above protocols, a diagnosis was achieved for 129 out of 174 embryos (74.12%). Of these, 55 embryos were diagnosed unaffected (42.6%) and 74 were diagnosed affected (57.4%). Eight embryos could not be diagnosed due to the biopsied cells indicating the presence of one parental genome only. In addition, twenty-seven embryos gave inconclusive results, as discussed in section 3.1.2.4, while no results were obtained from 10 embryos due to total amplification failure (5.7%) (figure 3.16).

The impact that a one-cell biopsy practice would have on the diagnosis rate was investigated by reanalysis of results from the cycles where protocol DM1/*APOC2*/D19S112 was used for diagnosis, as this was the protocol most likely to allow a one-cell diagnosis. One out of the 11 cycles where this protocol was used, was excluded because diagnosis in that case required results using two different protocols (table 3.10 and A2.5). Results indicated that if diagnosis was based on results from the first biopsied cell only, the diagnosis rate from these cycles would drop significantly from 73.4% to 53.1% ($p<0.05$, Fisher's exact test) (table 3.11).

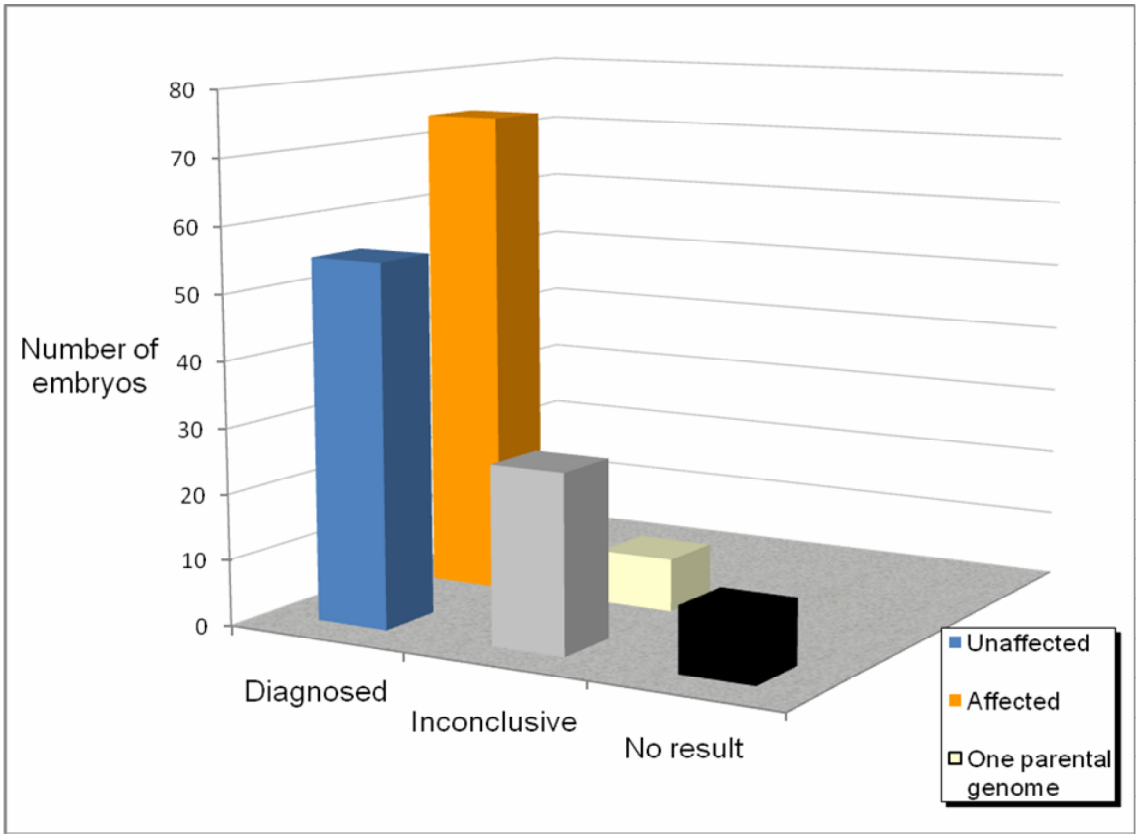

Figure 3. 16: Number of embryos diagnosed during PGD for DM1. 129 out of 174 embryos gave a clear diagnosis. Fifty-five embryos were unaffected and seventy-four were affected. Inconclusive results were due to one of the following: contamination, ADO, marker uninformativity, detection of one parental genome, result from one cell or other observations as indicated in table 3.15. (Embryos with result from one parental genome are shown separately).

Table 3. 11: Reanalysis of 10 DM1 PGD cycles to estimate diagnosis rate based on the results of a single (first-biopsied) cell

Patient number / Cycle	Number of embryos	Number of embryos rebiopsied	Cells biopsied-number of embryos			Diagnosis	Diagnosis based on result from the first biopsied cell
			1-cell	2-cell	3-cell		
10/1	4	2	3	1	0	3/4	1/4
11/1	12	0	2	10	0	8/12	6/12
12/1	3	0	0	3	0	1/3	1/3
11/1	15	0	3	7	5	12/15	8/15
15/1	6	0	5	1	0	1/6	1/6
19/1	2	0	0	2	0	2/2	2/2
22/1	7	2	2	5	0	7/7	4/7
19/2	5	0	0	5	0	5/5	3/5
22/2	5	0	3	2	0	4/5	4/5
19/3	5	0	1	4	0	4/5	4/5
Total	**64**	**4**	**19**	**40**	**5**	**47/64 (73.4%)**	**34/64 (53.1%)**

Table 3.12 indicates the number of cases where contamination was detected. This analysis excludes cases with TP-PCR amplification, where polymorphic marker analysis for contamination detection was not included in the protocol. A total of 260 single blastomeres were analysed. Contamination was detected in 8 out of the 260 cells (3%), and in 14 out of the 260 cell negative controls (5.4%) (overall 19/260, i.e. 8.46%). In three occasions (patient/cycle number 6/1, 11/1, 11/2) two PCR master mixes had to be prepared because of the high number of tubes. The PCR master mix negatives indicated a low level of external contamination in two cases.

Table 3. 12: Detection of contamination in PGD. The PGD results for each of these cycles may be found in appendix table A2.3-A2.7. External contamination was detected in 7/260 blastomeres. One blastomere (*) indicated maternal cumulus cell contamination and fourteen cell negatives indicated the presence of external contamination. Contamination was not detected in a cell and its corresponding negative control at the same time. Pre- (PCRM) and post-aliquoting (PCRN) negative controls of the PCR master mix, were obtained from all prepared master mixes.

Patient Number/ Cycle	Number of blastomeres	Cells with contamination	Cell negatives with contamination	PCRM1	PCRM2	PCR1N	PCR2N
1/1	8	0	0	-		-	
2/1	6	0	0	-		-	
4/1	14	0	1	-		-	
6/1	15	1	0	-	-	-	-
7/1	13	0	0	-		-	
8/1	12	0	3	-		-	
8/2	8	0	1	-		-	
8/3	6	1	0	-		-	
9/1	10	0	0	-		-	
10/1	5	0	0	-		-	
11/1	22	1*	2	-	-	-	-
11/2	32	2	4	cont	cont	cont	cont
12/1	6	1	0	-		-	
13/3	17	0	0	-		-	
15/1	7	0	0	-		-	
19/1	4	0	0	-		-	
19/2	10	2	2	-		-	
19/3	9	0	0	-		-	
20/1	8	0	0	-		-	
21/1	13	0	0	-		-	
22/1	12	0	0	-		-	
22/2	7	0	0	-		-	
23/1	6	0	0	-		-	
23/2	10	0	1	cont		cont	
Total	**260**	**8**	**14**				

3.1.2.2 Detection of cross-over

A cross-over event between DM1 and *APOC2* was observed during the analysis of the biopsied cells in one embryo from couple number 6. The maternal DM1 178bp allele was expected to be transmitted along with the 142bp *APOC2* allele, but in this case, the 125bp *APOC2* allele was detected instead. This suggested cross-over between the two alleles. Alternatively the findings could have occurred by the combination of maternal contamination with concurrent allele dropout of the *APOC2* 142bp and the *DMPK* 155bp products, though this is a highly unlikely occurrence. In any case, the paternal non-expanded 142bp allele was seen in the embryo along with one of the non-expanded maternal alleles (178bp) and the embryo was therefore considered as unaffected and suitable for transfer (figure 3.17).

Two embryos were transferred on day 4, including the embryo where cross-over was detected, leading to the establishment of a clinical pregnancy and the birth of a healthy male infant.

During PGD for couple number 10, a similar cross-over event was observed in one of the embryos diagnosed as unaffected (figure 3.18). The embryos were scored on day 5; a four-cell (with cross-over) and a five-cell embryo were transferred. A clinical pregnancy was established and a healthy child was born.

Buccal cells were collected from the babies born in both cases and tested using the DM1 triplex protocol. The results confirmed that, in both cases, the implanted embryos were the ones where the crossover event had been detected (figure 3.19).

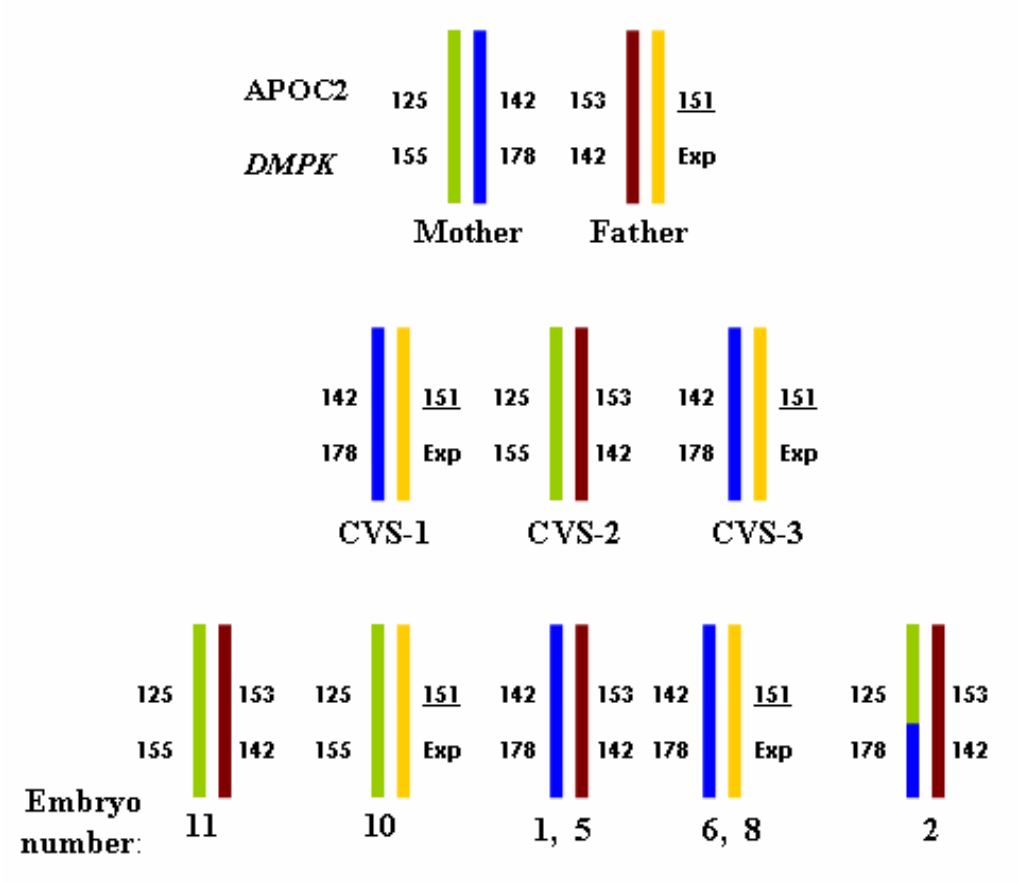

Figure 3. 17: Haplotype diagram for couple 6 (affected father) indicating a cross-over event detected in embryo 2 during diagnosis. Allele sizes for *APOC2* and DM1 are shown. CVS samples from previous pregnancies were available to allow detection of the phase allele (*APOC2* 151bp). Exp indicates the expanded allele which was refractory to PCR. Underlined alleles show the phase. Embryos 6, 8 and 10 were affected; embryos 1, 2, 5 and 11 were unaffected. Cross-over was detected between DM1 and *APOC2* in embryo 2. This was an unaffected embryo, showing the normal paternal alleles for both DM1 and *APOC2* regions however the 125bp *APOC2* maternal allele was detected instead of the 142bp allele due to cross-over. Embryos 4 and 7 showed inconclusive results while there was no result for embryo 3. (Kakourou et al., 2007)

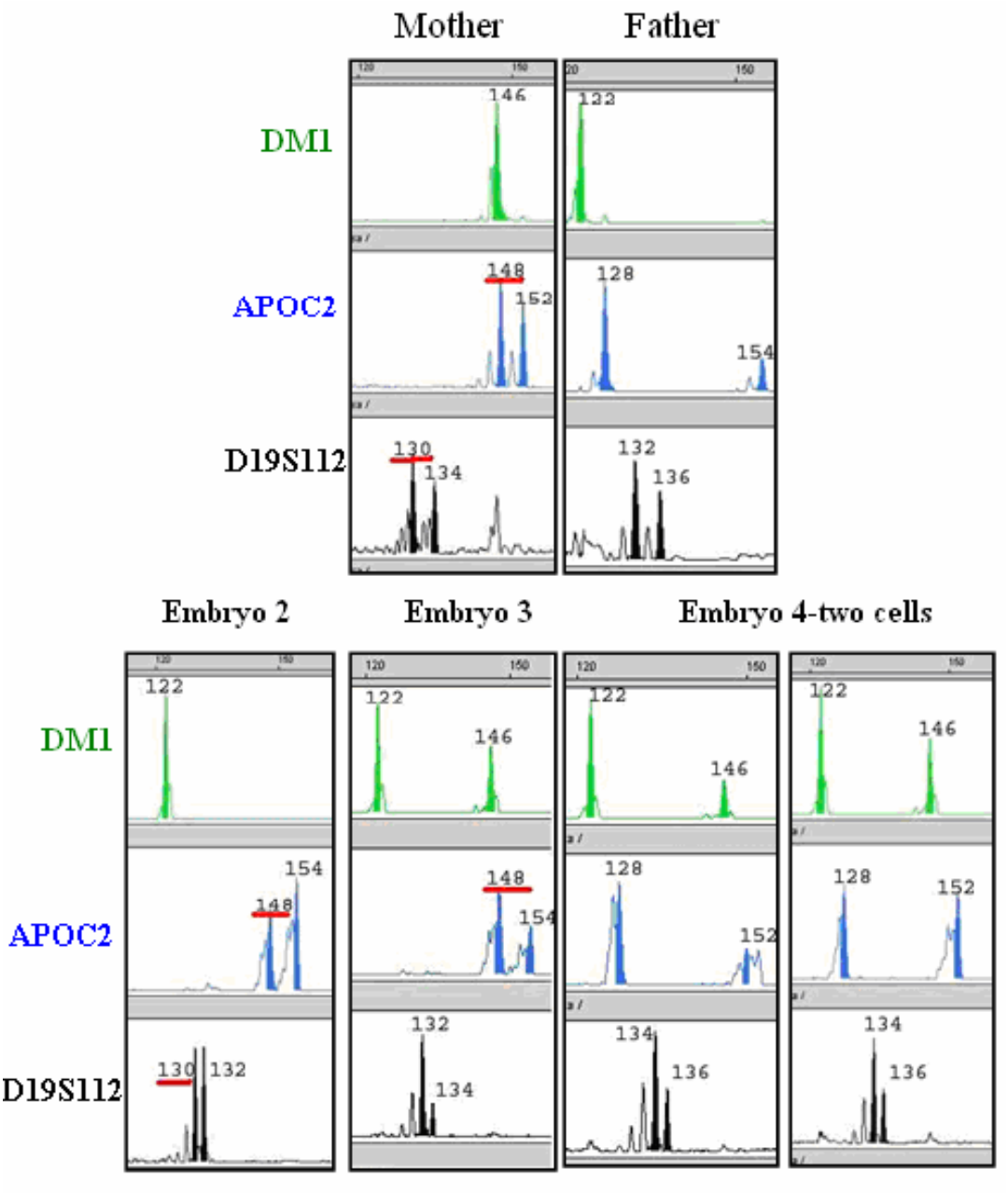

Figure 3. 18: PGD results for couple 10 (affected mother) where a cross-over event was detected in embryo 3. Underlined alleles indicate the phase. Embryo 2 was affected, embryos 3 and 4 were unaffected. Cross-over was detected between DM1 and *APOC2* in embryo 3. This was an unaffected embryo that showed the normal maternal alleles for both DM1 and D19S112 regions whereas the maternal disease-associated allele was detected for *APOC2* (Kakourou et al., 2007)

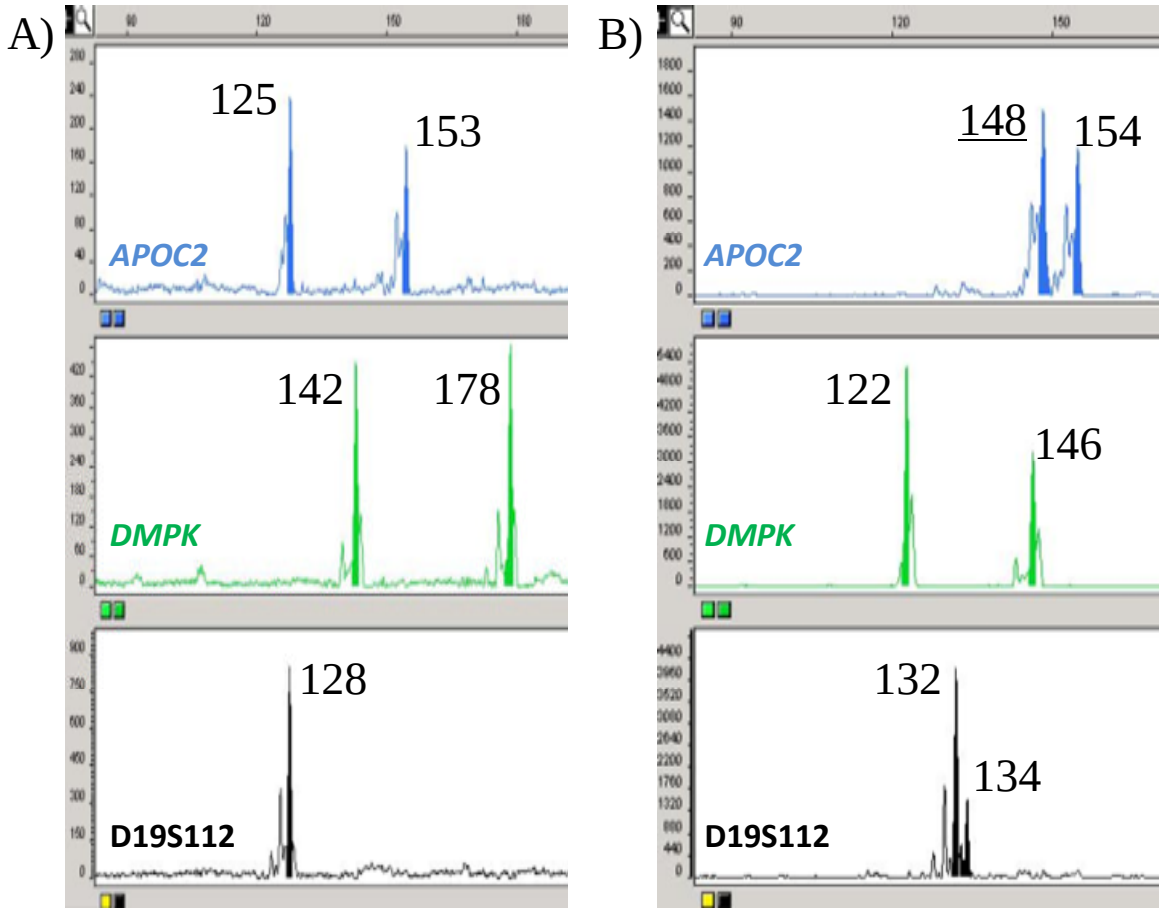

Figure 3. 19: Results from buccal cell analysis of two babies born following PGD. x-axis: PCR product size in bp, y-axis: fluorescence intensity (RFU). A) Buccal cell analysis from baby born following PGD for couple 6. The results correspond to the genotype of embryo 2, as indicated in figure 3.17 B) Buccal cell analysis from baby born following PGD for couple 10. The results correspond to the genotype of embryo 3, as indicated in figure 3.18.

3.1.2.3 Pregnancies

Thirty-six unaffected embryos were transferred into the mother's womb in 20 out of the 28 cycles. In 16/20 cycles resulting in an embryo transfer, two unaffected embryos were transferred, while in the remaining four cycles transfer of a single embryo took place. Embryo transfer was on day 4 for nine cycles and on day 5 for another nine cycles. For couple 16, embryo transfer was on day 6 and for cycle 2 of couple 23 the best quality embryo was transferred on day 4 and a second embryo was selected and transferred on day 5.

There were two additional embryo transfers, both for patient number 8. During the patient's second PGD cycle, all five biopsied embryos were diagnosed unaffected. Two embryos were transferred and two surplus unaffected embryos were cryopreserved. A pregnancy was not established, so the two cryopreserved embryos were thawed and transferred in a separate stimulation cycle, following which, implantation was, unfortunately, again unsuccessful. The same patient had a third treatment cycle, where she developed ovarian hyperstimulation syndrome (OHSS) after oocyte collection. The diagnosis was still completed, and the single embryo, diagnosed unaffected, was cryopreserved, then thawed and transferred in a separate treatment cycle, as before. Unfortunately an empty sac was detected at the 6 week scan. Another pregnancy was also lost as patient 23 miscarried in her second cycle at 7 weeks.

In case number 11, both of the transferred embryos implanted, therefore making the total number of implanted embryos eleven. The implantation rate, defined as the number of gestational sacs detected per total number of embryos transferred, was 28.2% (eleven out of thirty-nine embryos transferred overall). Seven pregnancies have led to the birth of healthy infants, in one case of twins and one pregnancy is ongoing. The clinical pregnancy rate per embryo transfer, defined as the number of gestational sacs and fetal heartbeat at 7 weeks detected per cycle with embryo transfer, was 40% (8/20) or 36.4% (8/22) respectively, including the two embryo transfers of frozen-thawed embryos, and the pregnancy rate per cycle to PGD was 28.57% (8/28) (table 3.13).

Table 3. 13: Summary of embryo transfers (ET) and outcome from DM1 IVF/PGD cycles
*cryopreserved/thawed unaffected embryos

Patient number/ cycle	No. of embryos transferred	ET Day	Pregnancy	Outcome
1	2	4	**Y**	Girl
2	0	n/a	n/a	
4	2	4	N	
6	2	4	**Y**	Boy
7	2	5	**Y**	Girl
8/1	0	n/a	N	
8/2	2	5	N	
	2*	new cycle	N	
8/3	hyperstimulation	n/a	n/a	
	1*	new cycle	**Y**	empty sac
9/1	0	n/a	n/a	
9/2	2	4	N	
10	2	5	**Y**	Girl
11/1	2	5	N	
11/2	2	5	**Y**	Boy and girl
12	1	4	N	
13/1	1	4	N	
13/2	0	n/a	n/a	
13/3	2	5	**Y**	Girl
15	0	n/a	n/a	
16	2	6	N	
19/1	2	4	N	
19/2	2	5	N	
19/3	2	5	**Y**	*Ongoing*
20	1	4	N	
21	0	n/a	n/a	
22/1	2	5	N	
22/2	1	4	**Y**	Girl
23/1	0	n/a	n/a	
23/2	2	4+5	**Y**	Miscarried at seven weeks

Seven out of the eleven embryos that implanted were at the morula to blastocyst stage, while the remaining four had 8 cells or fewer at the time of transfer. In two cases (number 6 and 10), the two embryos transferred had a different genotype and it was possible to identify which embryo had implanted by testing DNA from buccal cells of the baby born. In both cases, the embryos that implanted were the ones where a crossover event between *DMPK* and *APOC2* had been detected during diagnosis. For patient number 10, the 5-cell embryo that implanted had also been re-biopsied. Re-biopsied embryos implanted in another two cases (patient 13, cycle 3 and patient 23, cycle 2), though in one of them the patient subsequently miscarried at seven weeks (table 3.14).

Table 3. 14: Grade of implanted embryos In cases 6 and 10, it was possible to identify which of the two transferred embryos had implanted by testing DNA from the baby born. These are indicated in bold typing. In three cases, the implanted embryos had been rebiopsied (patients10, 13 cycle 3 and 23 cycle 2). M: morula stage embryo, or B: blastocyst stage embryo, *: rebiopsied embryo

Patient Number/Cycle	Embryos transferred (number of cells, grade)	Implanted Embryo/s
1	1) 6-cell, 2+ 2) 6-cell, 1- *	≤ 8-cell
6	1) 7-cell, 1- **2) 8-cell, 1-**	≤ 8-cell
7	1) Cavitating morula 2) Cavitating morula	M
10	**1) 5-cell, 1- *** 2) 5-cell, 1-, arrested	≤ 8-cell *
11/2	1) Blastocyst, 1 2) Hatching blastocyst	B, B
13/3	1) Hatching blastocyst * 2) Morula *	M or B *
8/3	1) Morula	M
22/2	1) 8-cell, 2+	≤ 8-cell
23/2	1) Morula * 2) Hatching blastocyst *	M or B * (miscarried)
19/3	1) Morula 2) Hatching blastocyst	M or B

3.1.2.4 Analysis of spare embryos for confirmation of diagnosis

Following PGD, most patients consented to the use of their spare embryos either for retesting and confirmation of diagnosis or for use in other research work.

Out of the 55 embryos diagnosed unaffected, 39 were transferred in 22 ETs and, nine out of the remaining sixteen embryos, unsuitable for cryopreservation, were retested for confirmation of diagnosis. Eight embryos were confirmed unaffected whereas one embryo gave an inconclusive result (detection of one parental genome). Similarly, forty-five out of the 74 affected embryos were retested, of which forty were confirmed affected, three gave an inconclusive result and two gave no result.

Moreover, twenty embryos with inconclusive results on diagnosis, five embryos with no result on diagnosis and seven embryos that had not been suitable for biopsy on day 3 were also tested following PGD. Results are summarised on tables 3.15 and 3.16.

In summary, including results from diagnosis and all follow-up testing, 150 embryos were diagnosed, of which 67 were unaffected (44.7%) and 83 affected (55.3%). The difference in the number of affected and unaffected embryos was not found to be statistically significant (binomial test, $p=0.22$).

Seven of the couples that had PGD (nine cycles) had previously experienced an affected pregnancy. From these couples, there were 44 embryos diagnosed, including analysis of the non-transferred embryos following PGD, of which 27 (61.4%) were affected and 17 (38.6%) unaffected. From all the remaining couples, that did not have a previous TOP or an affected child, there were 106 embryos, of which 56 (52.8%) were affected and 50 (47.2%) unaffected. In spite of a markedly higher number of affected embryos in the first group of patients, there was no statistically significant difference (NS) on analysis (Fisher’s exact test, $p=0.37$).

Table 3. 15: Reanalysis of embryos with inconclusive results during PGD *For two embryos where contamination was detected in one cell, the second biopsied cell indicated the presence of only one parental genome. ** "other": refers to the detection of odd/unexpected alleles, e.g. two paternal alleles

		Results from reanalysis					
Inconclusive results		Affected	Unaffected	No result	Inconclusive	Not Tested	Consent for other research
Observation	**Number of embryos**						
Result from one cell	1						1
Detection of one parental genome	8						8
Contamination*	5	2		1	2		
Allele Dropout	3		1		1	1	
Uninformative markers/ Shared alleles	14		3		7		4
Other**	4	3					1
Total	**35**	**5**	**4**	**1**	**10**	**1**	**14**

Table 3. 16: Summary of all spare embryo follow-up analysis following a PGD case. Forty out of the 55 embryos that were diagnosed unaffected were transferred following PGD and eight out of the remaining 15 embryos were reanalysed. The table indicates results from reanalysis of 86 embryos, 53 of which had received a diagnosis (45 affected/ 8 unaffected) during PGD. ET: embryo transfer.

	174 biopsied Embryos	Spares	Consent for other research	Not Tested	**Reanalysed Embryos**	Affected	Unaffected	No result	Inconclusive
Unaffected	55 (40 had ET)	15	5	2	**8**		7		1
Affected	74	74	24	5	**45**	40		2	3
Inconclusive	35	35	14	1	**20**	5	4	1	10
No Result	10	10	4		**6**	2	3	1	
Non-biopsied	-	7			**7**	2	5		

3.1.3 Summary of findings for section 3.1: PGD for DM1

- **Section 3.1.1: Protocol development**
  - Better amplification and lower ADO was achieved with ALB vs. PK lysis and with single lymphocytes vs. single buccal cells.
  - TP-PCR amplification on buccal cells was very inconsistent, indicating the impact of DNA quality on amplification.
  - TP-PCR can differentiate between the homozygous unaffected and affected samples but cannot give a clear diagnosis for the 5 CTG repeat homozygous samples.
  - Design of a new protocol, mTP-PCR, to overcome difficulties with scoring of the 5 CTG repeat homozygous samples. Along with amplification of the repeat region, this protocol provides additional information on contamination, ADO and the presence of the phase allele.
  - Clinical application in PGD for DM1, of two new optimised protocols, DM1 triplex and mTP-PCR.
- **Section 3.1.2: Results and follow-up from DM1 PGD cycles**
  - Embryo grade and quality of the biopsied cell (ie. anucleate cell, lysing cell, cell fragments) reflects amplification rate and overall diagnosis rate.
  - Higher diagnosis rate with two-cell vs. one-cell biopsy.
  - Detection of cross-over between DM1 and *APOC2* markers.
  - Overall encouraging diagnosis and pregnancy rates.
  - Analysis of spare embryos
    - No false positive or false negative results detected in follow-up study.
    - No statistically significant difference in the number of embryos diagnosed affected vs. unaffected, including results from diagnosis and follow-up analysis.
    - Higher percentage of affected embryos from parents with previously affected children but not reaching significance.

3.2 Investigation of DMPK repeat transmission

3.2.1 Determination of the repeat number by triplet-primed amplification (TP-PCR or mTP-PCR)

3.2.1.1 TP-PCR on genomic DNA

Unaffected individuals

Amplification of non-expanded alleles (of a maximum of 24 repeats) was generally consistent and allowed accurate sizing of the repeat, with some variation of ±1-2 repeats detected (figure 3.20).

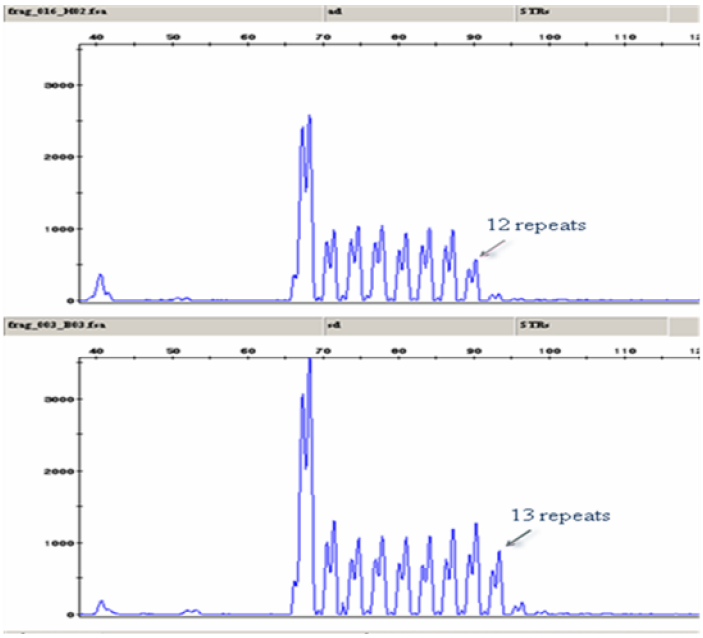

Figure 3. 20: Sizing of non-expanded DM1 alleles by TP-PCR. x-axis: PCR product size in bp, y-axis: fluorescence intensity (RFU). **T**op lane: genomic DNA TP-PCR amplification of a 12 CTG repeat homozygous individual. Bottom lane: genomic DNA TP-PCR amplification of a 13 CTG repeat homozygous individual. The first detected peak indicates 5 CTG repeats. The one CTG repeat size difference between the two samples is clearly visualized.

Affected individuals

Contrary to the accurate sizing of non-expanded alleles, when expanded repeats were amplified by either TP-PCR or mTP-PCR, the last sizeable peak of the diminishing ladder was variable both for genomic DNA and single lymphocytes. The pattern of triplet-repeat amplification was tested on a number of single lymphocytes from each

parent prior to PGD, in order to obtain the expected amplification “peak range” for the expanded repeat.

The triplet primed PCR protocols were also evaluated for their ability to discriminate between considerably different expansion sizes. Four control DNA samples were kindly provided by Dr. John Short (S.W. Thames Molecular Genetics Diagnostic Laboratory) from affected individuals with expansion sizes of 150, 550, 1100 and 1600 repeats. All sizes had been measured against a DNA ladder on a Southern blot. DNA samples from several of the DM1 patients, with known expansion size, were also analyzed along with the control DNA samples. Figure 3.21 gives an example of amplification from the four control DNA samples, in an attempt to detect some differences in amplification amongst the different expansions. Current analysis indicates that TP-PCR cannot be used to discriminate even between the strikingly different expansion sizes.

One consideration was that the different sizes of the non-expanded alleles from each affected sample could be influencing the pattern of amplification. It was, therefore, decided that only samples sharing the same non-expanded allele should be compared to allow detection of differences due to the presence of the expanded allele only. Five DM1 patients, where the expansion size was also known, shared a 122bp (5 CTG repeat) non-expanded allele. From all remaining samples, only two patients shared a 146bp non-expanded allele. Results from amplification for the 122bp/expansion patients are shown in figure 3.22. The graphs shown in figure 3.22 were also plotted against the log of the peak height in order to compare the slopes of the linear regression line (figure 3.23).

Modifications of the P2/P3R/P4CAG primer concentration ratio, as well as the PCR program conditions (shorter annealing and extension times), were attempted to encourage different amplification depending on repeat number but no differences were detected. Therefore no conclusions could be drawn from this analysis.

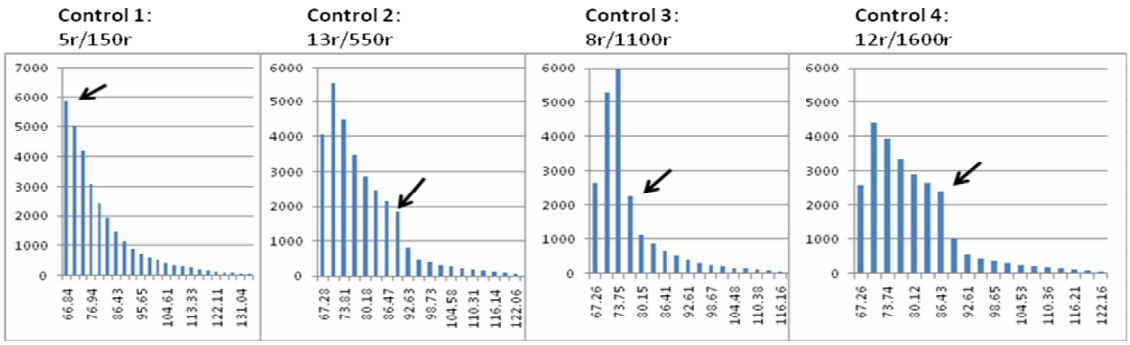

Figure 3. 21: Amplification of four control DNA samples of known repeat size. x-axis: PCR product size in bp, y-axis: fluorescence intensity (RFU). Arrows indicate the presence of the non-expanded allele in each case. The last sizeable peak on analysis was 131bp, 122bp, 116bp and 122bp for controls 1, 2, 3 and 4, respectively. This indicates that amplification is not relevant to the repeat size.

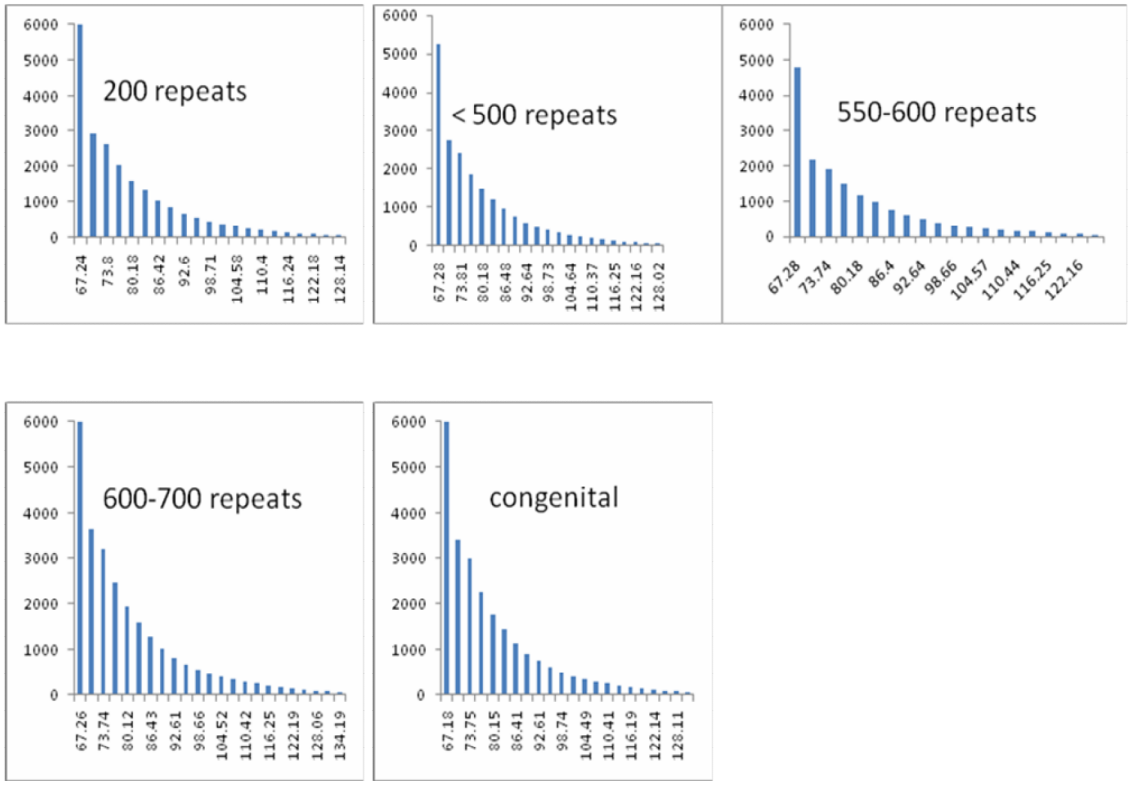

Figure 3. 22: Amplification from 5 DM1 patients, of known expansion size, sharing a common 122bp non-expanded allele. x-axis: PCR product size in bp, y-axis: fluorescence intensity (RFU). All samples, irrespective of expansion size, showed comparable triplet repeat amplification.

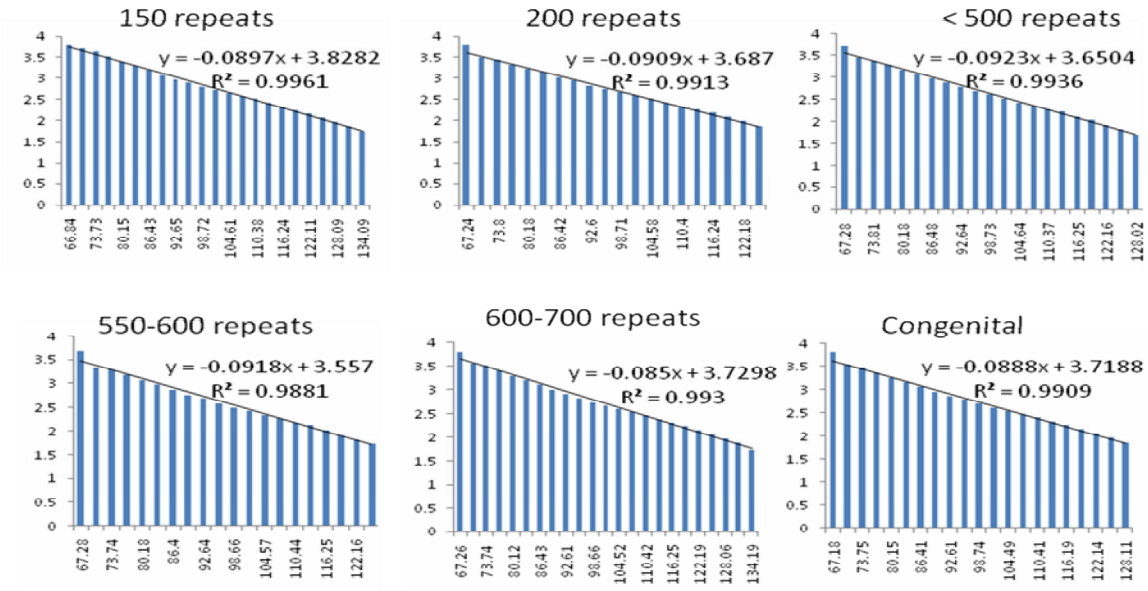

Figure 3. 23: Regression analysis of figure 3.22 TP-PCR amplification graphs. x-axis: PCR product size in bp, y-axis: fluorescence intensity (RFU). The 5/150 CTG repeat control was included in the analysis. All samples shared a 5 CTG repeat allele and had expanded alleles of different size. No differences were observed between the 6 samples, despite the differences in the size of the DM1 expansion.

3.2.1.2 TP-PCR single cell analysis

TP-PCR and mTP-PCR analysis was performed on single biopsied blastomeres from 11 PGD cycles (7 patients). The standard TP-PCR protocol was used for diagnosis in five cycles while the mTP-PCR protocol was used in the remaining six cycles. For two of the patients (number 8 and 13), both standard TP-PCR and mTP-PCR diagnosis was performed in different IVF/PGD cycles. Table 3.17 presents all fluorescent PCR blastomere readings, showing the size in base pairs (bp) of the last genetic analyzer sizeable peak of the ladder pattern, allowing comparison with results from amplification of single parental lymphocytes and parental genomic DNA. All cell readings presented in the table are from the 310 fluorescent genetic analyzer, so as to avoid differences in analysis due to machine variation. Analysis of fifty single cumulus cells was also performed for four of the patients (number 8, 13, 16 and 23) and results were comparable to amplification from single lymphocytes.

In the five cycles with standard TP-PCR diagnosis (indicated as 8/1, 9/2, 13/1, 13/2 and 16/1 on table 3.17), lack of amplification, that might indicate a 5 CTG homozygous sample, was taken as failure of amplification as this was the practice until application of the new mTP-PCR protocol.

Overall, all genomic DNA readings indicated a bigger product, i.e. more ladder peaks, compared to the single cell analysis. Readings from single affected lymphocytes were obtained from amplification of a minimum of 20 single cells, isolated and tested prior to each IVF/PGD case. These readings varied from cell to cell for the same individual, with the maximum difference detected being, on average, 52bp. The last peak of the TP-PCR ladder also varied from embryo- to embryo and from blastomere-to-blastomere.

Thirty-seven blastomeres were scored as unaffected. Most unaffected blastomeres gave a similar pattern of amplification between them, with a ± 1-2 CTG repeat difference detected in some cases. This was similar to amplification from single lymphocytes. In cycle 2 of patient 23, three single blastomeres from two different embryos showed a peak pattern unexpected for a 5 CTG homozygous sample, with the triplet repeat ladder pattern extending to 89bp.

Fifty-eight blastomeres showed the diminishing ladder pattern following TP-PCR or mTP-PCR, indicating an affected cell. In one cycle, the mTP-PCR protocol enabled detection of an embryo and a single blastomere showing amplification of one parental genome only (case number 21/1). The last detected peak of the ladder varied considerably between the affected blastomeres from an embryo, but was generally within the expected range as seen from amplification of single affected lymphocytes (patient specific analysis). Eleven blastomeres showed an increase in the size of the last detected fluorescent peak. In two out of the 58 blastomeres the product was over 50bp bigger than the biggest product detected in a single parental lymphocyte.

Follow-up analysis was performed on day 5 for two unaffected embryos (cycles 13/2 and 16/1) and nineteen affected embryos.
One of the unaffected embryos was tubed whole for amplification (patient 16), while the second embryo was disaggregated and two single blastomeres were isolated and amplified separately (cycle 2 of patient 13). Amplification from the embryo that was tubed whole and from one of the single blastomeres showed an increase in the number of CTG repeats by TP-PCR, of five and nine repeats respectively, compared to their day 3 result. This product was larger than the corresponding genomic DNA result.

Three out of the nineteen affected embryos that were available for testing, were tubed whole, a blastomere clump was isolated from another affected embryo and 31 single blastomeres were isolated from the remaining 15 embryos.

Fifteen of the single blastomeres, as well as the blastomere clump and the three embryos, showed an increase in the size of the last detected fluorescent peak compared to their day 3 result. In four of the single blastomeres the increase was over 50bp, compared to the maximum single lymphocyte parental size, while in a single blastomere from patient 9, the last peak was even larger than the genomic DNA amplification. Additionally, seven affected blastomeres showed a smaller ladder product on day 5 analysis, compared to the parental single lymphocytes, although the difference was less than 50bp in size.

Due to the inability of TP-PCR to accurately size the affected-range expanded alleles, as well as due to the association of poor cell quality with inferior triple-primed amplification, as detected mostly during buccal cell TP-PCR amplification, the significance of the above remains unclear.

Table 3. 17: TP-PCR blastomere results from 11 PGD cycles with follow-up analysis. The size of the last sizeable peak is indicated. Results from blastomeres of the same embryo are put in brackets. Arrows point to results from follow-up analysis. Bold type indicates unusual observations (red colour for follow up results), *: use of mTP-PCR, rather than TP-PCR, w:whole embryo, cl:clump, h, H:one or two-cell results respectively showing analysis from only one parental genome

Patient number/ Cycle	**Parental *DMPK* genotype**	**Unaffected genomic DNA (bp)**	**Unaffected Lymphocytes (bp)**	**Unaffected Blastomeres (bp)**	**Affected genomic (bp)**	**Affected lymphocytes (bp)**	**Range (bp)**	**Affected blastomeres (bp)**	**Non-biopsied embryos (bp)**
8/2	122/Exp 122/146	96-99	90-93	(93), (93,93)	189-224	147-189	42	n/a	93, 96 (190), (135)
8/3*		96	93-103	(68,68)	148	125-198	73	(116, 166), (172, 189)	
9/2	122/Exp 122/149	96-105	93-105	(96,99), (96,96)	225-258	113-195	82	(183, 199), (201), (189, **260**→ **300**)	
13/1	122/Exp 122/142	93-97	87-94	(87,90)	220-253	142-197	55	(176,194→ 110,194), (142,145→ 152,213) (152,155, 185→194)	
13/2		92-94	87-94	(89, 89→ 116, 89)	195-255	142-197	55	212, (128,156→ 120), (194→147,151,160,201,231) (104,128→ 144,160,163) (206,215→160) (188,208→131)	116 113
13/3*		90-93	89-93, av. 90	(68,68), (68,68) (92,92)	254-314	132-230	98	(170,223), (204,206)	
16/1	139/Exp 122/139	86	89-93	(90,90), (89,89→104w)	263	125-188	63	(149→ 124, 184, **249**), (133→**270**), (**260**→258w), (188,200→246cl) (204,208→252w), (180→258w) (170→131,154,**252**)	
20/1*	141/Exp 122/141	89	87-94	(86,86), (95), (86, 86)	198	129-162+236	33	(159, 162)	104,104 88,88
21/1*	144/Exp 122/147	95	92-101	n/a	224	137-185	48	(153,156), (127,171), (98^{h},150), (165,184), (144,165), ($160, 180)^{H}$	155,187
23/1*	122/Exp 122/122	68 (122hm)	68 (122hm)	n/a	147-295	170-192	22	(118,149→140,143,165), (148,154→143,180) (125,139→ 133,183)	
23/2*		68 (122hm)	68 (122hm)	(68,68),(68,68,**86,89**), (68,68,**86**)	253-272	125-133	8	n/a	n/a

3.2.1.3 Assessment of triplet repeat amplification following MDA

Amplification of the triplet repeat region from single cells following MDA was assessed in several lymphocytes and PGD spare embryos (table 3.18), in order to determine whether the whole genome amplification PGD methodology could offer any advantages in the diagnosis of DM1. Though only a few cells were obtained for MDA and post-MDA testing, i.e. three cells with the DM1 triplex protocol (protocol number 3) and eight cells with the mTP-PCR protocol (protocol 5), current results indicate generally poor amplification. A very high allele dropout rate was detected for both protocols used. Additionally, there seemed to be a bigger size product following triplet-primed amplification post-MDA, while in one cell there was a difference in sizing of the D19S112 marker allele, compared to sizing following standard PCR amplification. An aliquot of some of the MDA products was used for embryo sexing using AMELXY oligonucleotide pair (primer details in appendix table A1.1).

Table 3. 18: Single cell amplification post-MDA, using the DM1 triplex and mTP-PCR protocols. Allele sizes for patients 19 and 22 may be found in appendix table 3A.1. Patient 19 embryo results may be compared to amplification results as shown on table 3.7. Unexpected peak sizes are indicated in red colour. The TP-PCR column indicates the size of the last sizeable peak at the 310 genetic analyzer.

Patient	**Embryo number**	**Cell number**	**F-PCR readings**					
			TPPCR	**DM1**	**D19S112**	***APOC2***	**Diagnosis**	**XY Result**
DM1 patient 19	2PN/1	1		**ADO**/180	128/**ADO**	**ADO**/157	Unaffected	Male
		2	195	**ADO**/180	128/**ADO**			
	2PN/2	1		148/**ADO**	**ADO**/133	137/157	Unaffected	Male-**ADO**
		2		**ADO**/180	129/**ADO**	137/157	Unaffected	Male-**ADO**
		3	153	148/**ADO**	ADO/139			
		4	185	**ADO**/180	129/**ADO**			
	2PN/3	1	No result	No result	No result		Unaffected	Male-**ADO**
	2PN/4	1	183	124/180	128/130		Unaffected	Female
		2	Contamination in cell				Unaffected	Female
DM1 patient 22	1	1	173	141/168	128/130		Unaffected	Male
		2	176	**ADO**/168	128/130		Unaffected	Male

3.2.2 Study of allele transmission from follow-up analysis

3.2.2.1 Affected parent to embryo

In twenty-six PGD cycles where the mother was affected, there were clear results on diagnosis from 145 maternal transmissions. The non-expanded maternal allele was transmitted in 68 out of 145 embryos (46.9%), while the expanded allele was transmitted in the remaining 77 embryos (53.1%). In the remaining 2 PGD cycles, where the affected parent was the father, there were twelve embryos with diagnosis. The normal paternal allele was transmitted in 8 out of 12 embryos, and the affected allele was transmitted in the remaining four embryos.

The difference in the number of affected vs. unaffected embryos from an affected mother was not statistically significant (NS, binomial test, p=0.5). Additionally, transmissions from an affected father did not indicate a statistically significant difference between the number of expanded and non-expanded alleles detected in the embryos (NS, binomial test, p=0.38). It should be, however, stressed that only a small number of embryos were available for this analysis (twelve embryos).

3.2.2.2 Unaffected parent to embryo

Similarly, from 26 PGD cycles, there were 118 heterozygous transmissions from an unaffected father, where the smaller non-expanded allele was transmitted in 66 embryos (55.9%) and the larger allele in 52 embryos (44.1%) (NS, binomial test, p=0.23).

From the remaining two cycles, there were results from 8 heterozygous normal maternal transmissions, where the smaller allele was transmitted in three embryos and the larger allele in five.

If all cases are grouped together, regardless of individual DM1 status and parental sex, there were 283 transmissions where the smaller allele was transmitted in 145 embryos (51.2%) and the larger allele (larger normal or expanded allele) in 138 embryos (48.8%) (NS, binomial test, p=0.7).

Therefore, there was not any preferential transmission of either the smaller or larger alleles regardless of affected parent sex.

3.2.2.3 Grouping by repeat allele class

3.2.2.3.1 Sequencing of CTG repeat

The size of the DM1 expansion, estimated by Southern blotting, was known for only five of the affected individuals undergoing PGD. Primers DMPK2 and DMPK3 (appendix table A1.1) were used to amplify and sequence the *DMPK* repeat region of unaffected homozygous individuals. As indicated in figure 3.24, it was possible to compare the sequencing results with the results following amplification of the repeat region for these individuals, using primers DMPK1 and DMPK2. The size of the region outside the repeat, amplified by the DMPK1 and DMPK2 primers, was 107bp. Based on this, the size of the non-expanded alleles for all remaining patients and their partners was estimated using the formula:

Number of repeats= (PCR product size-107)/3.

Non-expanded alleles ranged from 5-24 repeats. Thirty-six out of the 69 (52.2%) non-expanded alleles had 5 CTG repeats, 27/69 alleles (39.1%) had 10-14 repeats, and 6/69 (8.7%) had other repeat size (figure 3.25).

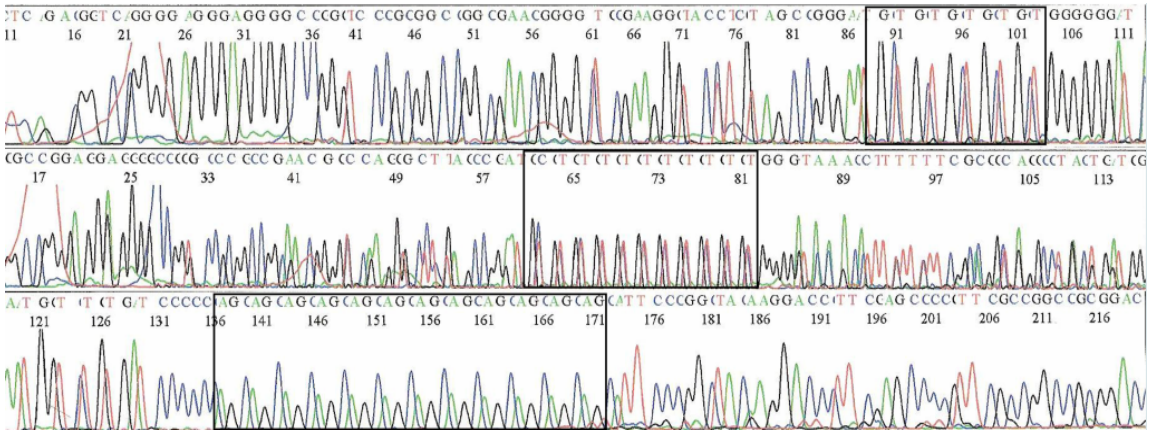

Figure 3. 24: Sizing of the non-expanded DM1 alleles by sequencing using primers DMPK2 and DMPK3 The y-axis indicates the level of fluorescence emitted by the incorporation of a nucleotide and the x-axis shows the order in which the nucleotides were added. Lane 1: 5 CTG repeats, Lane 2: 11 CTG repeats, Lane 3: 12 CAG repeats (lane 3 sequenced from reverse strand). When DNA was amplified from each of these unaffected homozygous individuals using primers DMPK1 and DMPK2, the size of the PCR product was 122bp, 140bp and 143bp respectively. Based on this, and depending on the PCR product size following DMPK1/DMPK2 amplification, it was possible to estimate the number of repeats in the non-expanded alleles for each individual, whether unaffected heterozygous or affected.

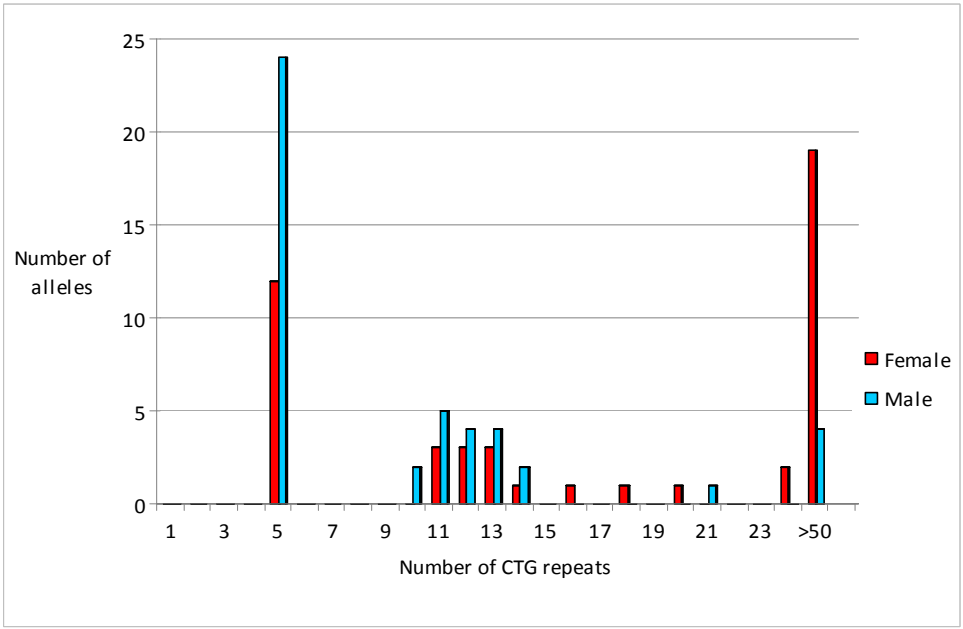

Figure 3. 25: Distribution of DM1 CTG repeat alleles amongst the referred couples. Affected individuals are indicated in the >50 CTG repeat category. The distribution of non-expanded alleles is bimodal with most alleles having 5 repeats and a second mode at 10-14 repeats.

Repeat alleles were grouped into the following categories: (A) 5-11 repeats, (B) 12-18 repeats, (C) 19-37 repeats and (D) > 50 repeats (Imbert et al., 1993). For the investigation of allele transmission, every embryo where some indication for allele transmission could be identified was included, even when diagnosis was incomplete.

3.2.2.3.2 Maternal transmissions

From the 16 couples where the female partner was affected, 10 females (17 cycles) were of genotype A/D, four females (4 cycles) were of B/D genotype and 2 females (5 cycles) were of genotype C/D.

There were 96 transmissions from A/D females, leading to 42 unaffected (43.75%) and 54 affected embryos (56.25%) (NS, binomial test, p=0.26). For class B/D, allele B was transmitted in 6 out of 19 embryos and allele D was transmitted in the remaining 13 embryos (NS, binomial test, p=0.17). Finally, out of 30 transmissions in class C/D the non-expanded allele (C) was transmitted in 20 embryos and the expanded allele in 10 (NS, binomial test, p=0.09).

One of the unaffected female partners was a class A/A homozygous, while the other female had a B/C genotype. In this case allele B was transmitted in 3/8 and allele C in 5/8 embryos (figure 3.26).

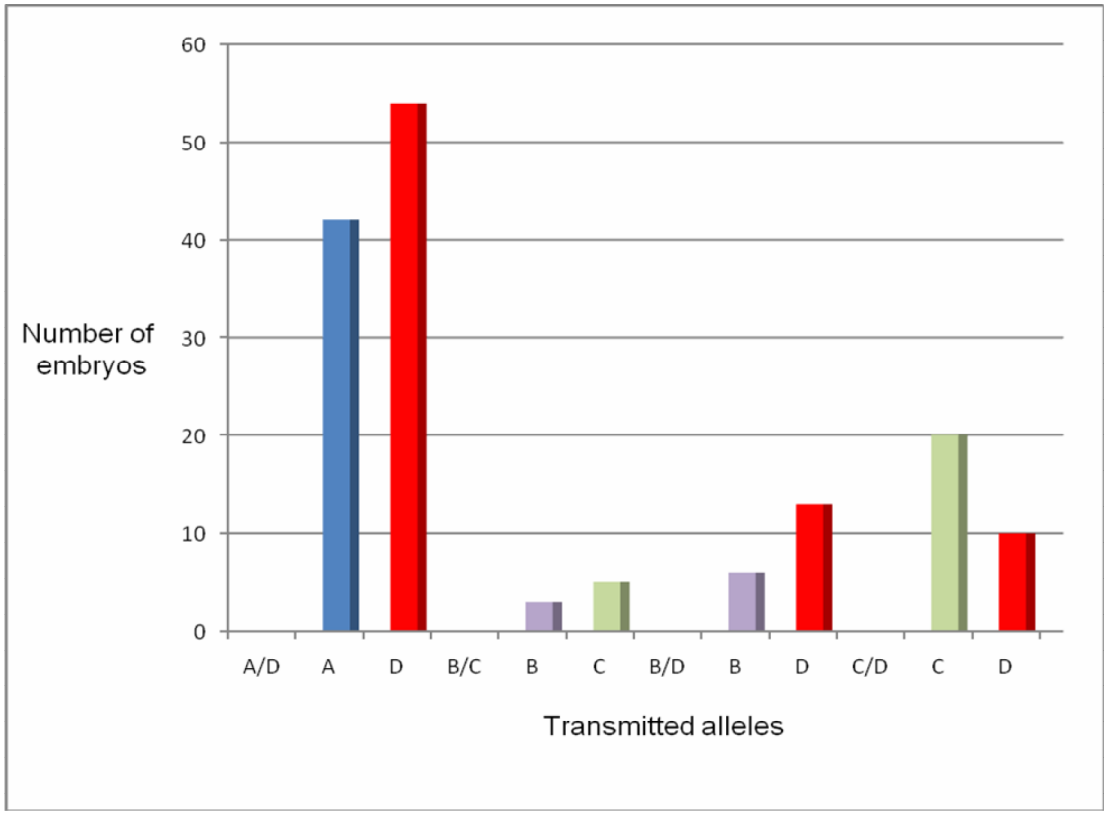

Figure 3. 26: Transmissions of repeat alleles from mothers of the genotype: A/D (10 females-96 transmissions), B/C (1 female- 8 transmissions), B/D (4 females- 19 transmissions) and C/D (2 females-30 transmissions)

3.2.2.3.3 Paternal transmissions

There were 16 couples where the male partner was unaffected. In three of the couples (4 cycles) the unaffected partner was homozygous for class A repeat alleles (5 repeats). In 5 couples (6 cycles), the male was heterozygous but both alleles were in the 5-11 repeat class (genotype A/A heterozygous). In five out of the six cycles, the protocol used allowed detection of the smaller allele transmission in 11 embryos, whereas the large allele was transmitted in 13 cases. Eight unaffected males (16 cycles) were of genotype A/B. Allele A was transmitted in 55 out of 94 embryos (58.5%) and allele B in the remaining 39 embryos (41.5%) (NS, binomial test, p=0.12).

The genotypes of the two affected males, patient number 12 and 6, were A/D and B/D respectively. Allele transmissions were 2/3 allele A and 1/3 allele D for patient 12, 6/9 allele B and 3/9 allele D for patient 6 (figure 3.27).

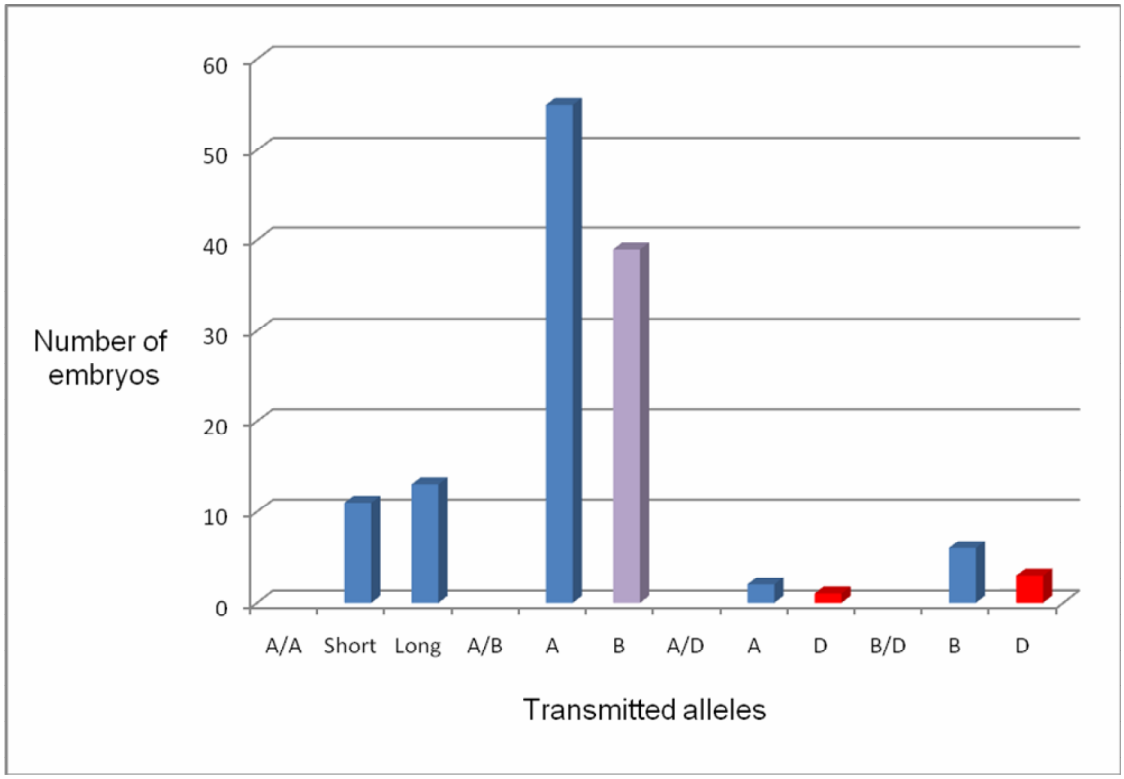

Figure 3. 27: Transmissions of repeat alleles from males of genotype: A/A heterozygous (5 males- 24 transmissions), A/B (8 males- 94 transmissions), A/D (1 male- 3 transmissions), and B/D (1 male- 9 transmissions)

3.2.2.4 Affected and unaffected embryo development

Embryos were scored from day 2 to day 4 or 5 of development. For the purposes of comparing the preimplantation development of affected *vs*. unaffected embryos, all rebiopsied embryos and all embryos biopsied later than day 3 were not included in this analysis, so that all embryos investigated had undergone exactly the same procedures. Some embryos were scored both on day 4 and day 5, while other embryos were excluded from this analysis as they had not been morphologically assessed. Data is summarised in tables 3.19 and 3.20 and figures 3.28 and 3.29.

A general trend for better development for the affected embryos can be observed. The group of embryos that had two cells biopsied on day 3 and had been assessed on day 4, table 3.19, indicated a similar number of affected and unaffected embryos. Comparison of the development of these embryos showed that more affected embryos developed to the morula or cavitating morula stage by day 4 compared to the number of unaffected embryos at the same stage (9/26 *vs.* 1/25) ($p<0.05$, Fisher's exact test).

Table 3. 19: Day 4 scoring of affected and unaffected embryos with one, two or three cells biopsied on day 3. A statistical analysis was performed for the embryos with two cells biopsied. There was a statistically significant difference in the number of affected embryos growing to the morula/cavitating morula stage, compared to the number of unaffected embryos (Fisher's exact test).

		Number of embryos at each developmental stage DAY 4 SCORING				
Number of diagnosed and scored embryos	**Number of cells biopsied - Embryo diagnosis**	**2-5 cell**	**6-8 cell**	**9 cell-premorula**	**Morula/ cavitating morula**	
	1 cell					
5	Affected	3	1	1		
3	Unaffected	3				
	2 cells					
26	Affected	5	4	8	9	p<0.05
25	Unaffected	5	16	3	1	
	3 cells					
4	Affected	1	2		1	
3	Unaffected	1	2			

The biggest number of embryos where day 5 scoring was available came from the group that had two cells biopsied on day 3. The majority of these (36/54) were affected and 18/54 were unaffected. More affected embryos in the 2-cell biopsy group developed to the morula and blastocyst stage (21/36, 58.3%) compared to the number of unaffected embryos (6/18, 33.3%) but this difference was not statistically significant (Fisher's exact test, p=0.15). More data is, however, necessary to allow a more comparable analysis between the two groups.

Similarly, three out of the four affected embryos that had three cells biopsied on day 3, had become morulae/blastocysts by day 5, compared to 5 out of the 10 unaffected embryos that had three cells biopsied. Although the numbers are small, the current analysis indicates that there is no statistically significant difference between the two groups (Fisher's exact test, p=0.58).

Table 3. 20: Day 5 scoring of affected and unaffected embryos with one, two or three cells biopsied on day 3. Comparison of the affected and unaffected embryos that had two or three cells biopsied on day 3 and grew to the morula/cavitating morula or blastocyst/hatching blastocyst stage by day 5 was performed, however no statistically significant difference was detected for any group (Fisher's exact test).

		Number of embryos at each developmental stage DAY 5 SCORING				
Number of diagnosed and scored embryos	**Number of cells biopsied -Embryo diagnosis**	**2-5 cell**	**6-8 cell**	**9 cell-premorula**	**Morula/ cavitating morula**	**Blastocyst/ Hatching blastocyst**
	1 cell					
3	Affected		1		1	1
1	Unaffected	1				
	2 cells					
36	Affected	8	5	2	13	8
18	Unaffected	4	2	6	5	1
	3 cells					
4	Affected		1			3
10	Unaffected	3	2		2	3

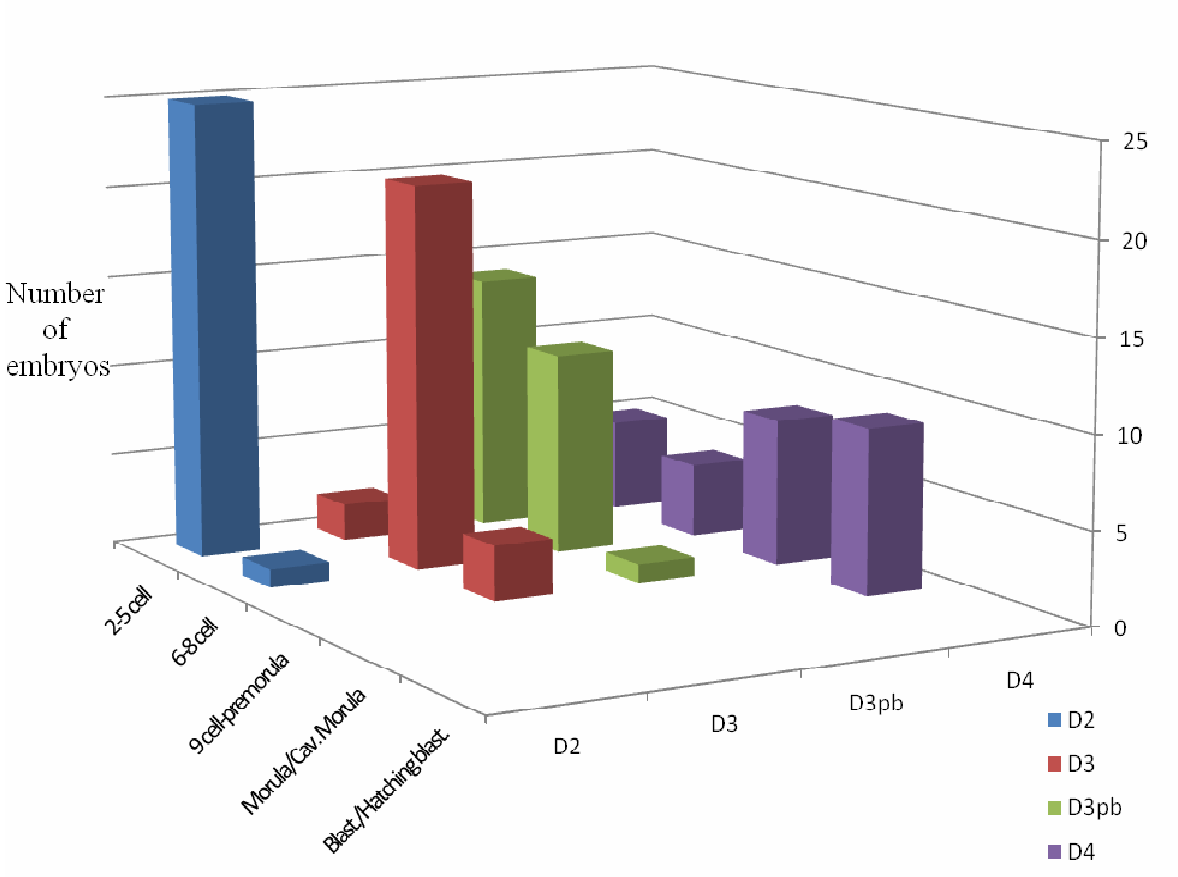

Figure 3. 28: Day 2 (D2) to Day 4 (D4) scoring of affected embryos at the 2-5 cell stage, 6-8 cell stage, 9cell-premorula, morula/cavitating morula and blastocyst/hatching blastocyst stage. D3pb: D3 post-biopsy

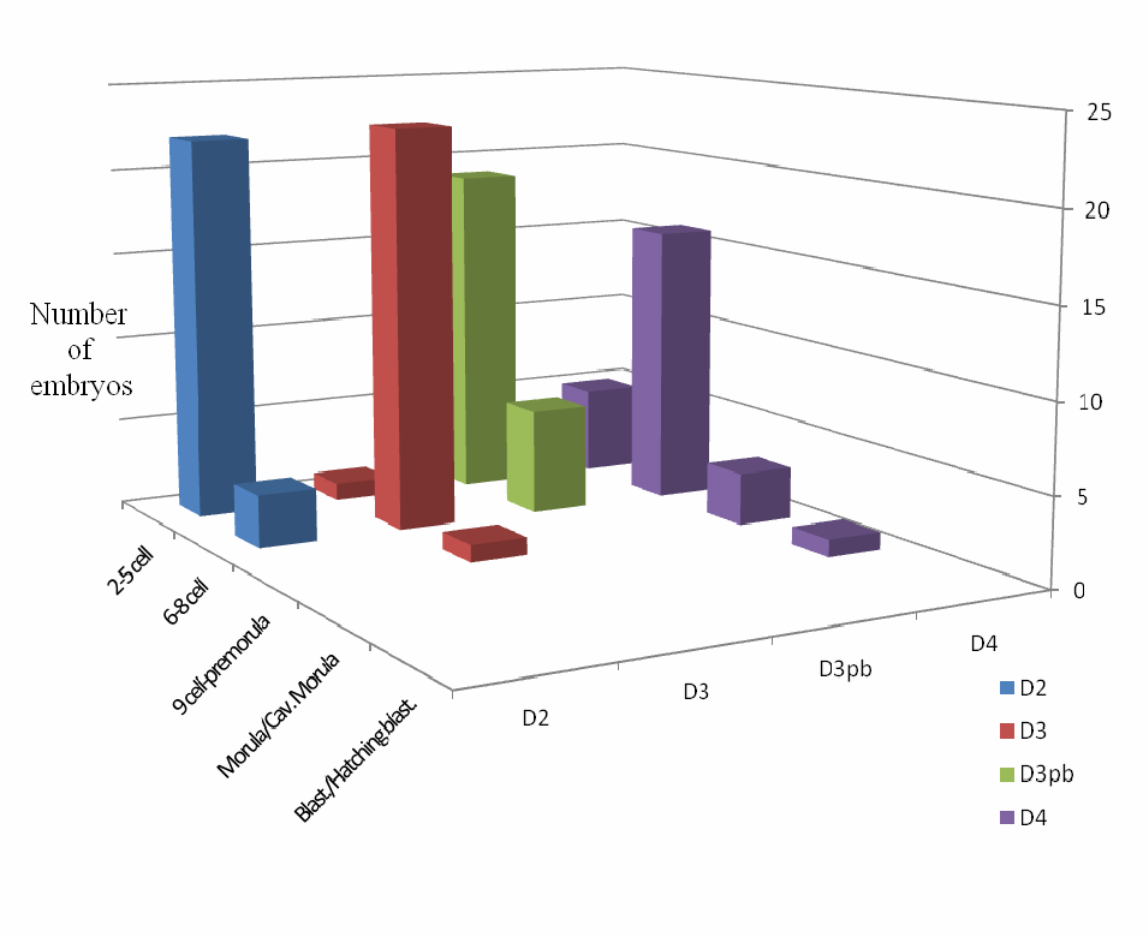

Figure 3. 29: Day 2 (D2) to Day 4 (D4) scoring of unaffected embryos at the 2-5 cell stage, 6-8 cell stage, 9cell-premorula, morula/cavitating morula and blastocyst/hatching blastocyst stage. D3pb: D3 post-biopsy

3.2.3 Summary of findings for section 3.2: *DMPK* repeat transmission

- **Section 3.2.1: TP-PCR sizing**
    - TP-PCR allows accurate sizing of non-expanded repeat alleles (up to 24 repeats).
    - Inability of TP-PCR to differentiate between even strikingly different expansions.
    - Inconsistent amplification of expanded repeats both on genomic DNA and single cells.
    - Blastomere quality has an effect on TP-PCR amplification.
    - Potential expansion of the triplet repeat was suggested on analysis of blastomeres on day 3, compared to amplification from single lymphocytes, as well as on analysis of blastomeres from the same embryo between day 3 to day 5 of development.
    - Poor TP-PCR amplification and high ADO rates were observed following MDA.

- **Section 3.2.2: *DMPK* allele analysis in embryos**
    - No preferential transmission of expanded *DMPK* alleles.
    - No overall preferential transmission of either smaller or larger alleles, regardless of affected parent sex or repeat size.
    - More affected than unaffected embryos developed to the morula/cavitating morula stage by day 4 (no impact of the number of cells biopsied on embryo development).

3.3. Expression work

3.3.1. Sample processing

3.3.1.1 RNA Isolation results

The procedure for extraction of RNA from single cells was initially optimised using lymphocyte clumps and single lymphocytes. RNA isolation, reverse transcription and cDNA amplification was performed as described in sections 2.4.2.1 and 2.4.2.2. Successful cDNA amplification was achieved from single lymphocytes for *ACTB* and for a lymphocyte pellet for *DMPK* (figure 3.30).

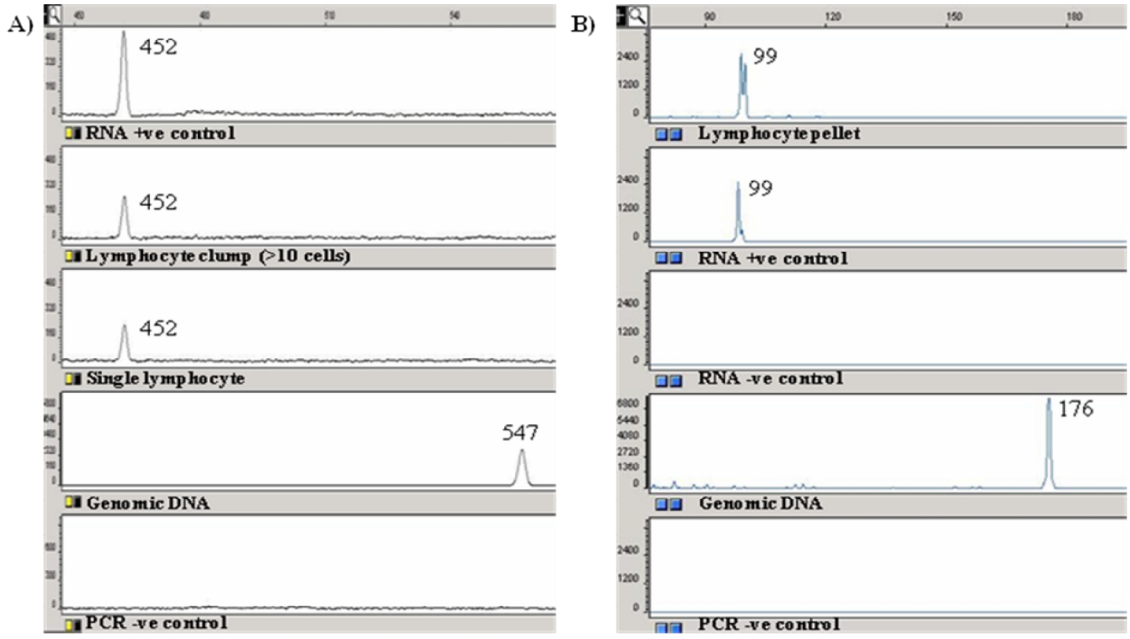

Figure 3. 30: cDNA amplification from lymphocyte clumps and single lymphocytes. A) *ACTB* amplification was achieved for lymphocyte clumps and single lymphocytes B) *DMPK* amplification was only achieved when the whole lymphocyte pellet, following lymphocyte extraction from blood, was used. HeLa RNA was used as a positive control. Negative controls included a reverse transcription negative (RNA –ve), which included RNA but where no reverse transcriptase was added, and a PCR negative, from the cDNA PCR amplification, where no cDNA was added in the tube (methods 2.4.2.2). The RNA and DNA product sizes are indicated in base pairs (appendix table A1.3). +ve: positive, -ve: negative.

Oocyte and embryo samples for microarrays

RNA was isolated from each oocyte and embryo sample (MIIa-MIIe, Ba-Bd and DMa) using the AllPrep DNA/RNA Micro kit (Qiagen Ltd., UK) (Methods section 2.4.2.1) and eluted in 14µl of water. The concentration and integrity of eluted RNA was measured by loading 1µl on a Eukaryote Total RNA Pico Series II chip on the Agilent Bioanalyzer 2100 (table 3.21, figures 3.31 and 3.32).

Agilent Bioanalyzer analysis can indicate RNA degradation, genomic DNA contamination, or the presence of other contaminants, by the absence of 28S and/or 18S peaks, skewing of the 28S peak or the presence of additional peaks or spikes respectively. The 28S and 18S peaks were detected in all samples, however, they were lower for the oocytes because of the lower concentration of RNA isolated from these samples. RNA contamination or fragmentation products were not detected in any of the samples. High RIN numbers were given by the Agilent Bioanalyzer for all of the embryo samples, indicating very good RNA quality (methods section 2.5.5.4).

Table 3. 21: Agilent Bioanalyzer RNA concentration readings for each oocyte and embryo sample prior to RNA amplification. RIN numbers were assigned by the software for all of the embryos and two of the oocyte samples only, possibly because of the lower RNA concentration of the oocytes.

OOCYTES			**EMBRYOS**		
	RNA concentration (pg/µl)	**RIN number**		**RNA concentration (pg/µl)**	**RIN number**
MIIa	78	6.2	Ba	393	10
MIIb	65	n/a	Bb	191	9.5
MIIc	48	n/a	Bc	63	8.7
MIId	50	n/a	Bd	246	10
MIIe	52	7	DMa	120	9.3

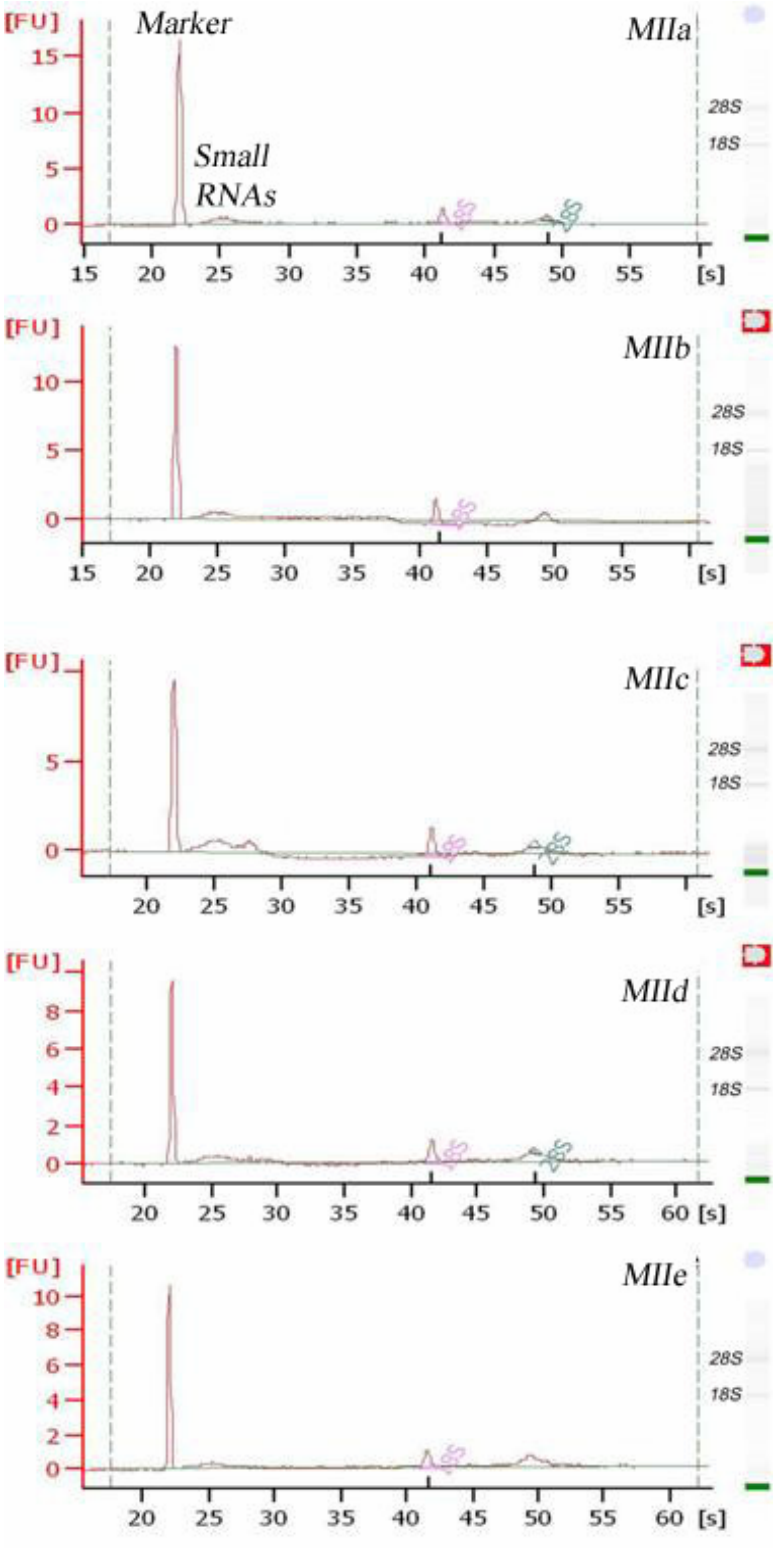

Figure 3. 31: Electropherograms and gel-like images indicating RNA integrity for oocytes MIIa-MIIe, using the Agilent Bioanalyzer. The visual signs of the 28S and 18S ribosomal units, at the electropherogram (left) and gel-like image (right) are most important in assessing the quality of RNA. No contamination was detected in samples MIIa-MIIe. The small peak at around 24-29s (100bp) indicates small RNAs, (shown on the first lane) such as 5S, 5.8S subunits and tRNAs.

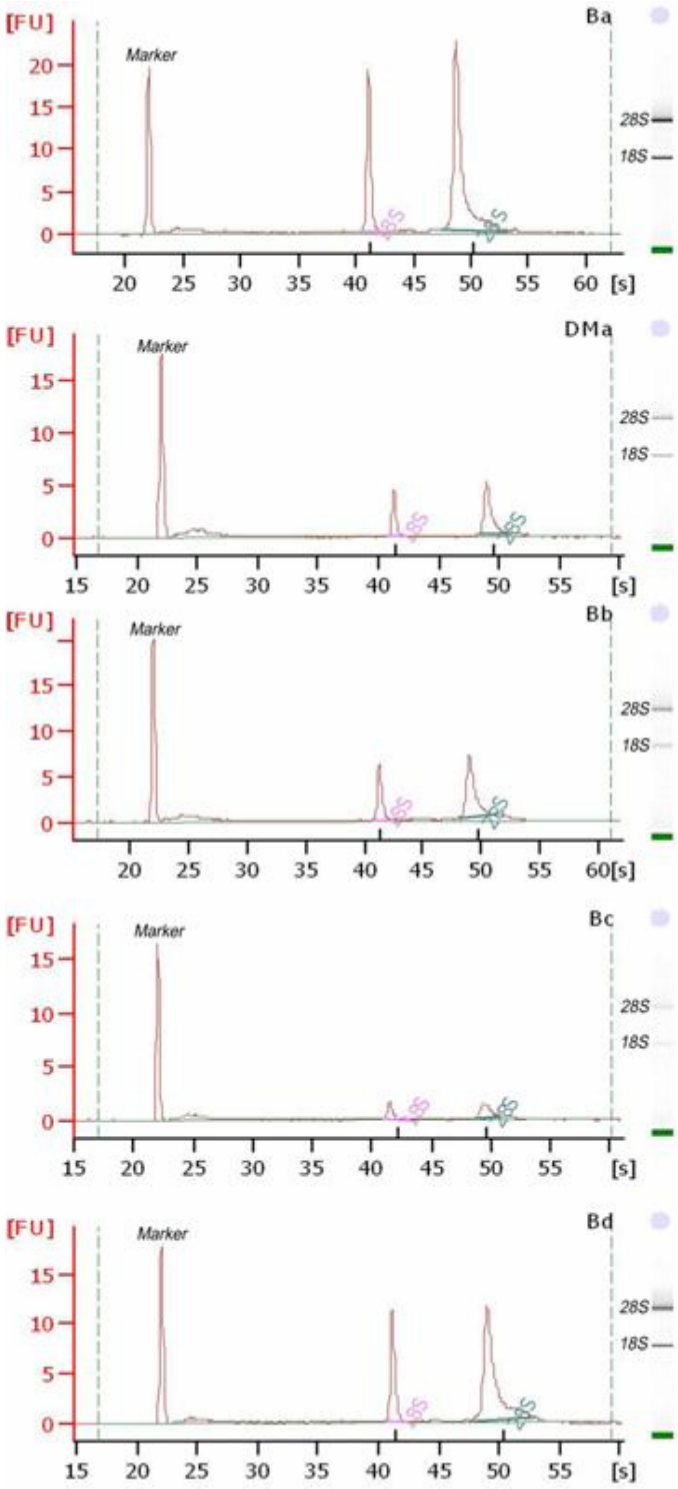

Figure 3. 32: Assessment of RNA Integrity using the Agilent Bioanalyzer for blastocyst stage embryos Ba-Bd and DMa. The 28S and 18S ribosomal units are clearly detected and no degradation or contamination can be visualized.

3.3.1.2 RNA Amplification results

Nine microlitres of each oocyte and embryo RNA sample was converted into labelled cRNA by a two-round *in vitro* transcription using the Applied Biosystems, NanoAmp RT-IVT Labeling kit (section 2.4.2.3). The concentration and integrity of the labelled cRNA was tested on the NanoDrop® ND-1000 Spectrophotometer as well as on the Agilent Bioanalyzer using the Eukaryote Total RNA Nano Series II chip (table 3.22). The Nano chips were loaded quickly and the working area was thoroughly cleaned. Bioanalyzer and agarose gel images of amplified cRNA are shown in figures 3.33 and 3.34.

Table 3. 22: Concentration of labelled cRNA samples (Nanodrop and Bioanalyzer readings).

	RNA concentration				
	Nanodrop	**Bioanalyzer**		**Nanodrop**	**Bioanalyzer**
	ng/µl	**ng/µl**		**ng/µl**	**ng/µl**
OOCYTES			**EMBRYOS**		
MIIa	41.76	36	Ba	59.46	51
MIIb	22.89	18	Bb	126.56	91
MIIc	296.12	292	Bc	232.83	207
MIId	140.18	134	Bd	1115.3	1027
MIIe	206.48	184	DMa	88.26	70

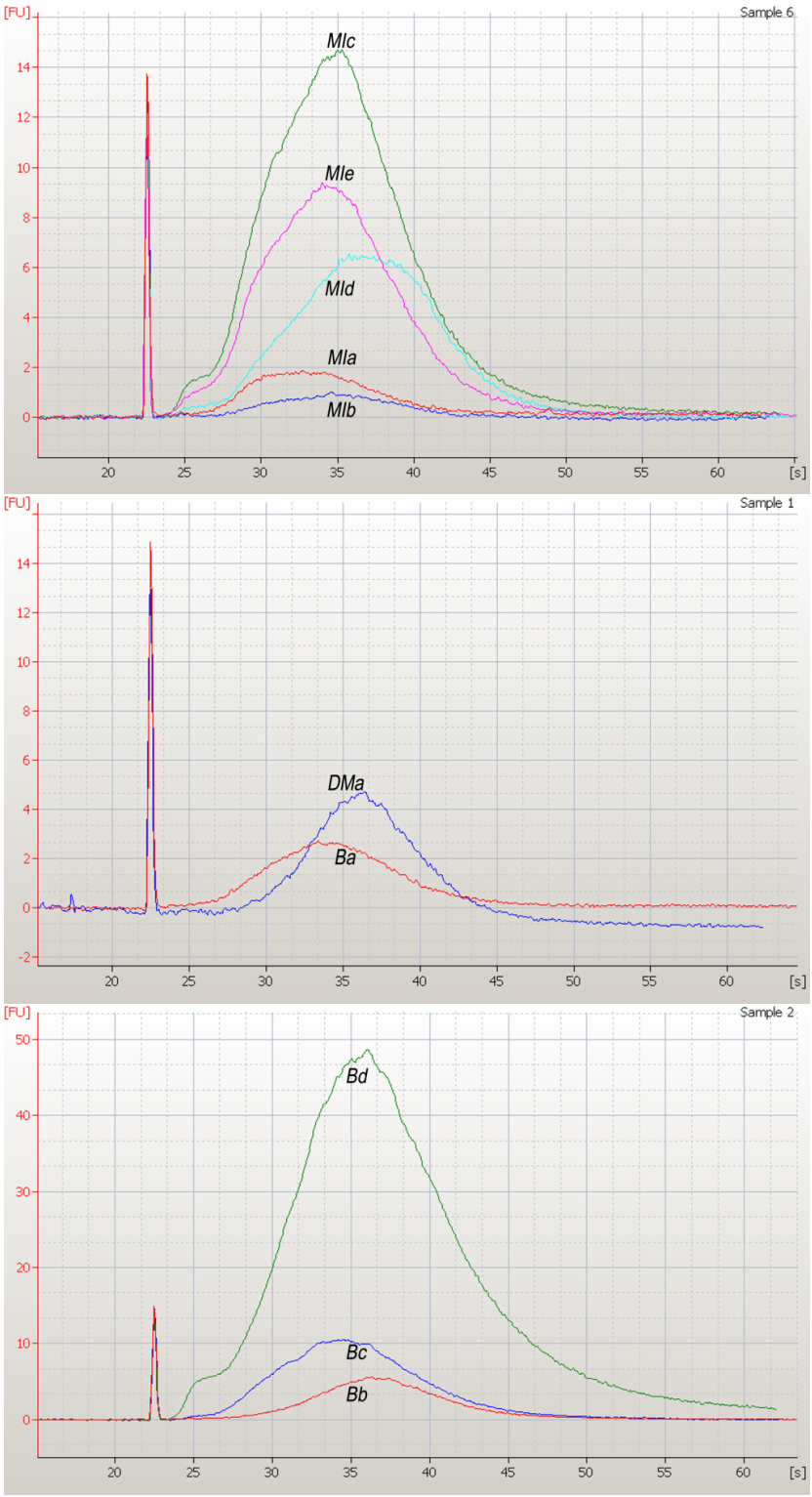

Figure 3. 33: Electropherograms of amplified cRNA on the Bioanalyzer Nanochip. A broad hump was seen starting at approximately 25 seconds for all samples, diminishing between 40-50 seconds.

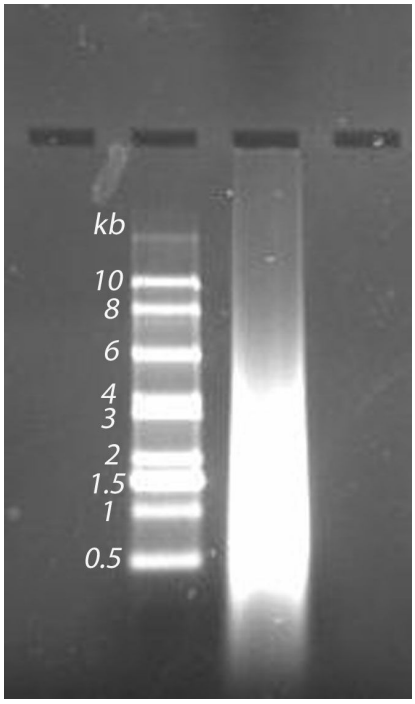

Figure 3. 34: Example of agarose gel analysis of amplified cRNA. Image indicates a 1.5% agarose gel stained with ethidium bromide. Amplified cRNA was run alongside a 0.5-10kb RNA Ladder (Invitrogen, UK). A smear was seen starting from <500bp.

Samples MIIa and MIIb that gave the lowest concentration readings after two rounds of amplification were pooled together into 186µl of sample MIIa+b (93µl each) and concentrated down to approximately 95µl by vacuum centrifugation (Concentrator plus, Eppendorf UK Limited). The concentration of the new sample MIIa+MIIb was 54.06ng/µl.

3.3.1.3 Microarray hybridization

The recommended amount of labelled cRNA for hybridization on the array is 10 µg. Table 3.23 indicates the total yield in microgram (µg) of labelled cRNA for each sample from a final volume of 95µl based on the nanodrop readings of table 3.22 (5 out of the 100µl of the eluted sample were kept separately for agarose gel and/or concentration assessment).

10 µg, the recommended amount, was available for three oocyte and three blastocyst samples and was used to perform hybridizations in triplicate for each sample type.

Five microgram of labelled cRNA was hybridized for samples MIIa+b, Ba and DMa.

Table 3. 23: Total RNA yield (µg) for each amplified sample available for hybridization on the microarray. Shaded areas indicate a yield lower than the recommended 10µg.

OOCYTES	cRNA yield (µg)	EMBRYOS	cRNA yield (µg)
MIIa+MIIb	5.14	Ba	5.65
MIIc	28.13	Bb	12.02
MIId	13.32	Bc	22.12
MIIe	19.62	Bd	105.95
		DMa	8.38

The following microarray experiments were performed (patient/sample details as in section 2.3.3.2):

- MII oocyte triplicates
  - MIIc
  - MIId
  - MIIe
- Blastocyst stage embryo triplicates
  - Bb
  - Bc
  - Bd
- Three microarray experiments were performed for samples MIIa+b, Ba and DMa, by hybridizing 5µg of labelled cRNA due to the lower yield achieved.

Following scanning and analysis, the Expression Array System software of the Chemiluminescent genetic analyzer produced heat map images, where the processed samples were grouped based on the similarity of the detected signals (figure 3.35).

Additionally, hierarchical clustering analysis provided a visual representation of the data, illustrating in a dendogram (tree graph) the grouping of genes based on the similarity between them. The gene clusters that are closest to each other are combined into a higher-level cluster (Quackenbush, 2001) (figures 3.36, 3.37).

The level of expression was also indicated by colouring based on the $\log_2$ (ratio), as described in methods section 2.5.6. Log_2 values close to zero were coloured black, values greater than zero (up-regulated genes) were coloured red and values lower than zero (down-regulated genes) were coloured green. The intensity of the colour also represented the level of differential expression.

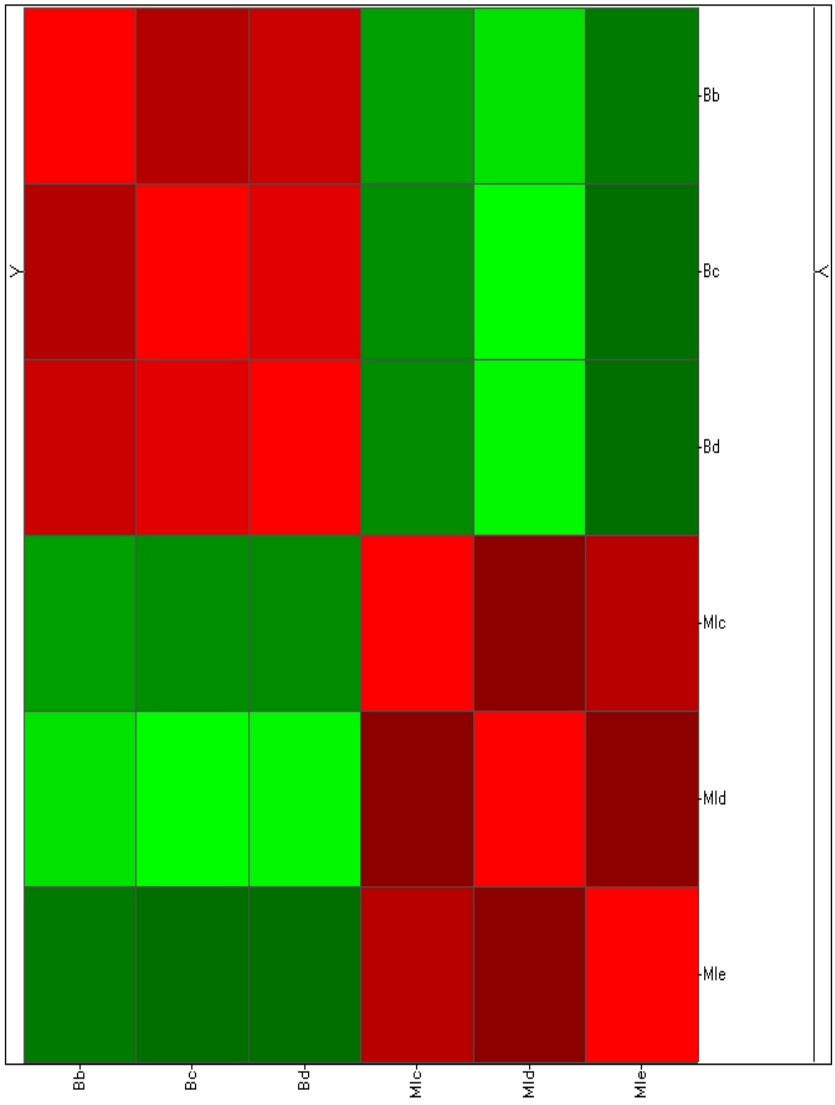

Figure 3. 35: Heat map for triplicates of oocytes and embryo samples of over 10 µg yield. Colour coding indicates the degree of similarity. Red colour is a sign of high similarity, with bright red representing the highest degree of similarity between two samples, while green colour indicates dissimilarity. As seen in the image, embryo samples Bb, Bc and Bd were successfully identified as being similar between them (red squares). The same applies for the oocyte samples, MIIc, MIId and MIIe (here labelled MIc, MId and MIe).

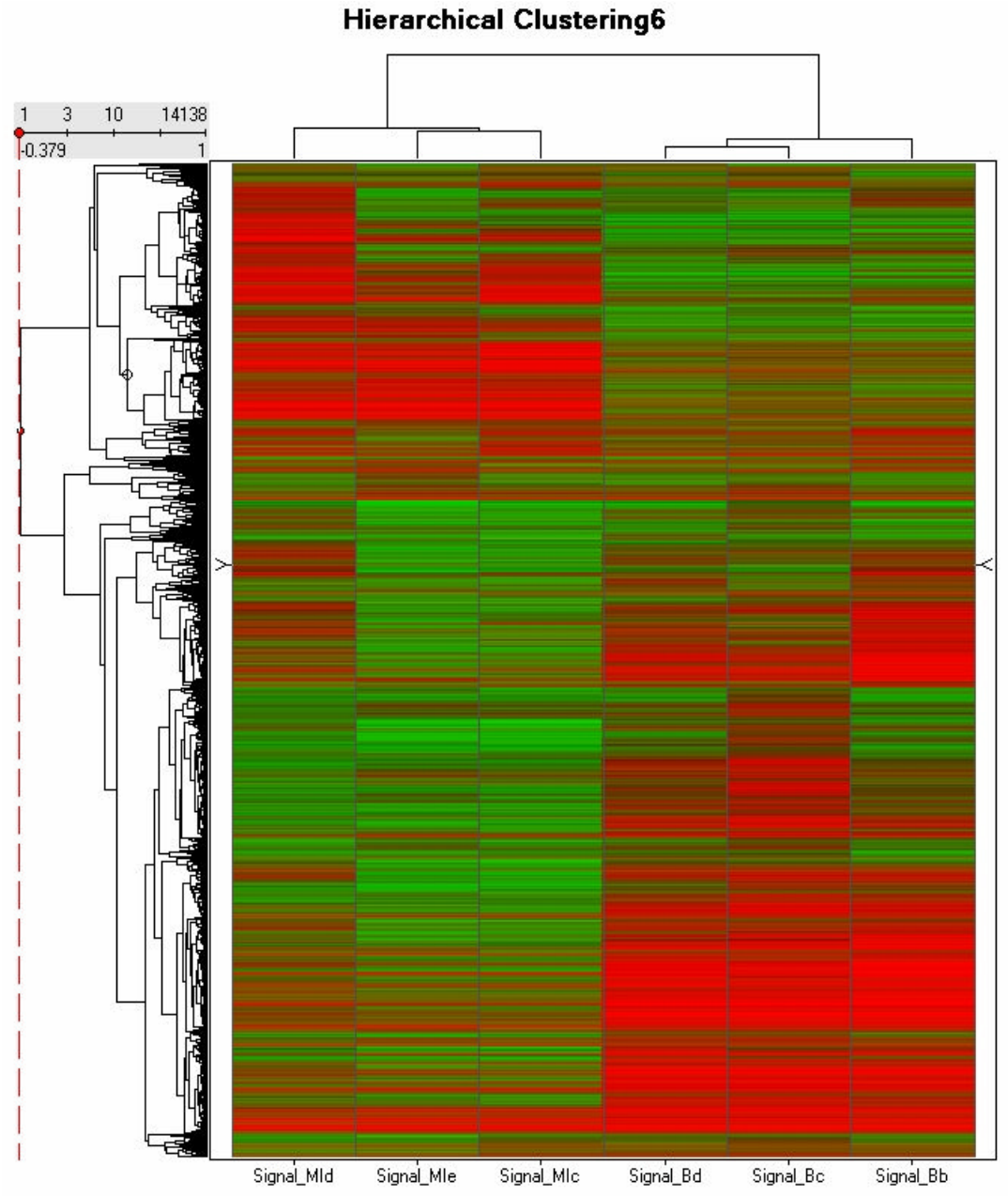

Figure 3. 36: Hierarchical clustering analysis of oocyte and embryo (>10µg yield) replicate array experiments. The dendogram shows a grouping of all genes based on the similarity between them. The oocyte and blastocyst samples were grouped separately by the software. Red colour: upregulated genes. Green colour: downregulated genes. The level of up- or down-regulation is reflected by the intensity of the colour. Samples MIIc, MIId and MIIe were noted MIc, MId and MIe, when setting the sample list for analysis and are indicated as such in this figure produced by the instrument.

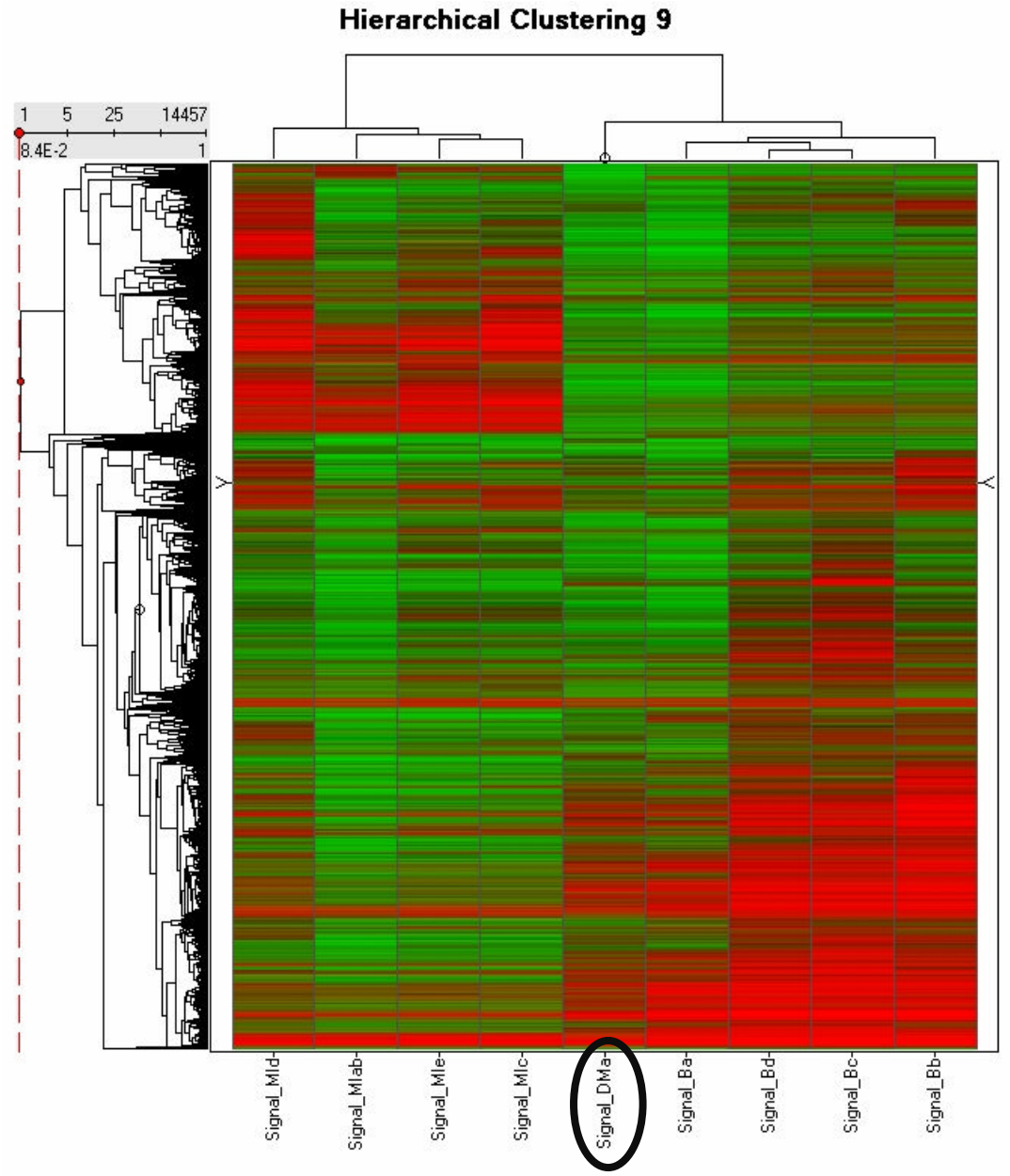

Figure 3. 37: Hierarchical clustering analysis of all array elements (addition of samples MIIab (labelled MIab in figure), Ba and DMa, to the previous dendogram). Overall, all oocyte and embryo samples were grouped separately. The DM1 affected embryo sample (circled) showed the least similarity compared to the remaining embryo samples.

3.3.2 Gene expression profiling of human oocytes and embryo blastocysts

3.3.2.1 Determining the level of gene expression

The oocyte and blastocyst samples were analyzed independently and genes in each sample type were classified into groups of high, medium and low expression. In order to calculate the cut-offs for each category, the top and bottom 5% of all detected signal values were eliminated at first, so as to avoid any outlier values. Following that, the number of detected probes was equally split into three groups according to the signal value as indicated in table 3.24.

Table 3. 24: Oocyte and blastocyst signal intensity units for high, medium and low expression level

Expression level	Oocyte signal Intensity units (probes/ genes)	Blastocyst signal Intensity units (probes/ genes)
High	>22686 (4088/3870)	> 29526.36 (4591/4303)
Medium	6016.72-22681.77 (3504/3326)	7254.45-29525.33 (3935/3700)
Low	< 6015.22 (4087/3788)	< 7253.86 (4592/4282)

3.3.2.2 Assessment of microarray results

The level of expression of cumulus cell-specific genes, kit ligand (*KITLG*), steroidogenic acute regulator (*STAR*), gremlin1 (*GREM1*), prostaglandin-endoperoxide synthase 2 (*PTGS2*), pentraxin-related gene (*PTX3*), amphiregulin (*AREG*) and epiregulin (*EREG*) was investigated in order to exclude contamination from RNA belonging to cumulus cells (Kocabas et al., 2006;Menezo, Jr. et al., 2007).

KITLG, *STAR*, *GREM1*, *PTX3*, *AREG*, and *EREG* were not detected in any of the oocyte or embryo replicates. *PTGS2* was also absent from the oocyte samples and gave a very low signal (841 intensity units) in only one of the embryo replicates (Bd).

Further initial analysis involved the investigation of several genes with known expression levels in oocytes and embryos, as reported by other investigators, as a way of assessing the accuracy of these results. For example, the results confirmed higher expression of programmed cell death 5 (*PDCD5*) and adenomatosis polyposis coli (*APC*) in oocytes compared to blastocysts (fourteen and four times higher respectively), and higher expression of H2A histone family, member Z (*H2AFZ*) and beta actin (*ACTB*) in the blastocyst samples, compared to the oocytes (showing 180 times and 13 times fold change respectively). In addition, *RB1* was not detected in the oocyte samples, but showed medium expression level (12700 intensity units) in the blastocyst samples (Dobson et al., 2004;Wells et al., 2005b).

3.3.2.3 Global characteristics of human oocyte and human blastocyst gene expression

The total number of probes detected after sorting the microarray data by signal-to-noise ratio and flags, as described in Methods section 2.5.6, were 14662 (MIIc), 16821 (MIId) and 15062 (MIIe) for the oocyte samples and 15652 (Bb), 17654 (Bc) and 16011 (Bd) for the embryo samples. Only genes where probes were consistently detected in all three replicates of a sample were taken as expressed. Overall, an average signal value from three replicates was available for 11679 probes for the oocytes and 13118 probes for the blastocyst samples.

The genes detected in the oocyte and blastocyst samples were mapped to the PANTHER database biological process categories and compared to the list of genes available on the microarray (using gene expression tools of the PANTHER database). The proportion of genes belonging to the protein biosynthesis, protein metabolism and nucleic acid metabolism categories was greater than statistically expected in both sample types ($p<0.001$). Other significantly over-represented categories included cell cycle, DNA metabolism, DNA repair, DNA replication, oxidative phosphorylation, translational regulation, mRNA splicing, rRNA metabolism ($p<0.001$).

The oocyte and blastocyst genes were listed by level of expression. The 50 genes with highest expression in oocytes and blastocysts are listed in tables A3.1 and A3.2 of appendix 3. These were investigated in more detail as indicators of the most important processes involved in each developmental stage. A summary of the most represented

biological function categories from the fifty genes of highest expression is shown in table 3.25. Oocytes and blastocysts shared only eight probe IDs, within the fifty genes of highest expression (table 3.26).

Table 3. 25: Classification by biological process of 50 genes with highest gene expression in the human MII oocyte and human blastocyst

	Percentage of genes	
Biological Process	**Oocyte**	**Blastocyst**
Biological process unclassified	27.9	6.9
Protein metabolism and modification	26.5	69
Nucleoside, nucleotide and nucleic acid metabolism	11.8	5.2
Cell cycle	10.3	-
Transport	4.4	-
Cell proliferation and differentiation	4.4	3.4
Developmental processes	2.9	-
Intracellular protein traffic	2.9	5.2
Cell structure and motility	2.9	1.7
Carbohydrate metabolism	1.5	1.7
Homeostasis	1.5	-
Immunity and defense	1.5	6.9
Oncogenesis	1.5	-

Table 3. 26: List of genes shared between the top 50 of highest signal in both oocyte and embryo samples.

Gene Symbol	Gene Name	Biological process	Oocyte Signal Position	Blastocyst Signal Position
FLJ40448	Unassigned	Unclassified	5	13
GPR103	G protein-coupled receptor 103	G-protein mediated signalling	7	24
RPLP1	ribosomal protein, large, P1	Protein biosynthesis	9	14
HNRPA1	heterogeneous nuclear ribonucleoprotein A1	mRNA splicing	12	26
RPL7A	ribosomal protein L7a	Protein biosynthesis	15	34
Unassigned	Unassigned	Biological process unclassified	33	29
LOC440055	Unassigned	Protein biosynthesis	52	28

3.3.2.4 Differential expression

There was a statistically significant difference in gene expression ($p<0.05$) between oocytes and blastocysts for 5243 probes (4910 genes). The majority of the differentially expressed genes were of unclassified biological function (37.3%) followed by genes belonging to the protein metabolism and modification class (18.7%), and the nucleic acid metabolism class (16.7%).

Overall, 2591 probes were underexpressed, and 2652 probes were overexpressed in the blastocyst compared to the oocyte (based on $\log_2$ ratios, as previously described). Most genes with higher expression in the blastocyst belonged to the protein metabolism and modification class, with the majority involved in protein biosynthesis. On the other hand, genes showing higher expression in the oocyte sample were mostly involved in nucleic acid metabolism, mainly mRNA transcription (figures 3.38-3.40).

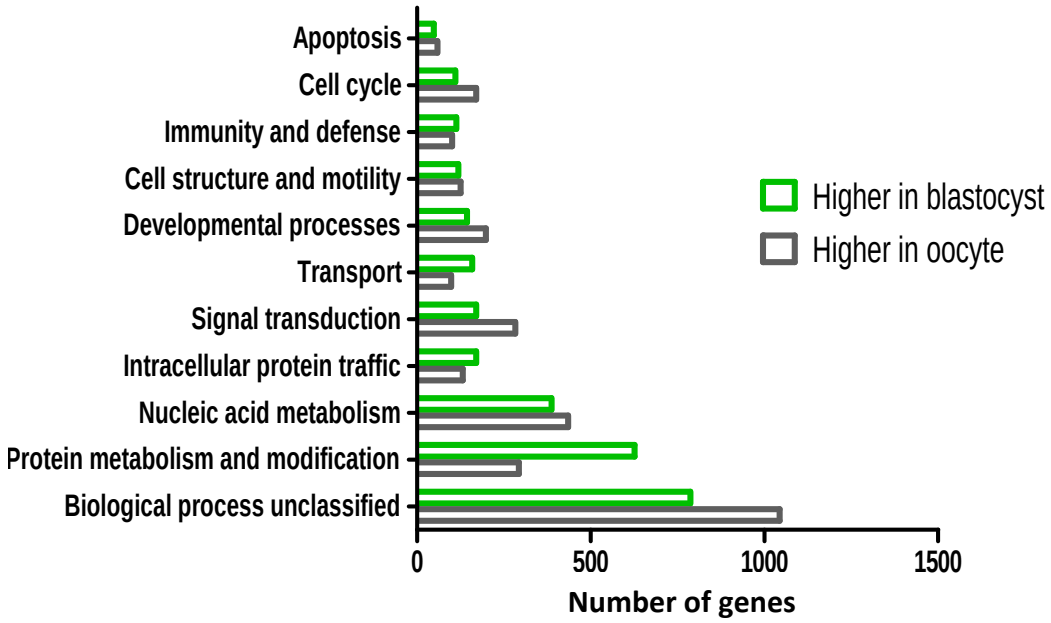

Figure 3. 38: Genes differentially expressed in the blastocyst compared to the oocyte sample ($p<0.05$). Genes were identified using the Celera dataset and grouped per PANTHER biological process category.x axis: number of genes, y axis: PANTHER biological process

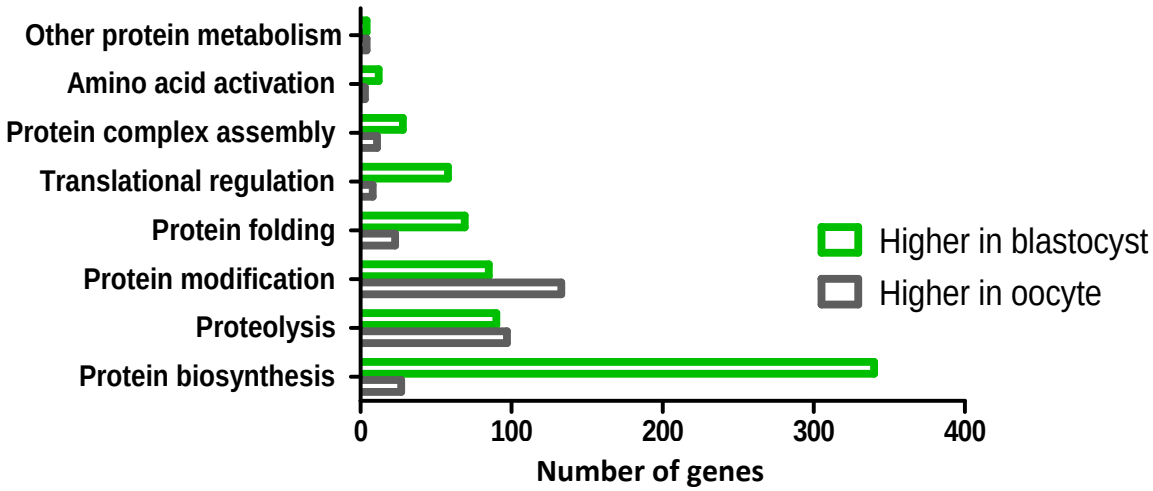

Figure 3. 39: Sub-categories of the protein metabolism and modification biological process. Most genes showing higher expression in the blastocyst are involved in protein biosynthesis. Most protein modification genes were downregulated at the blastocyst stage. x axis: number of genes, y axis: PANTHER biological process

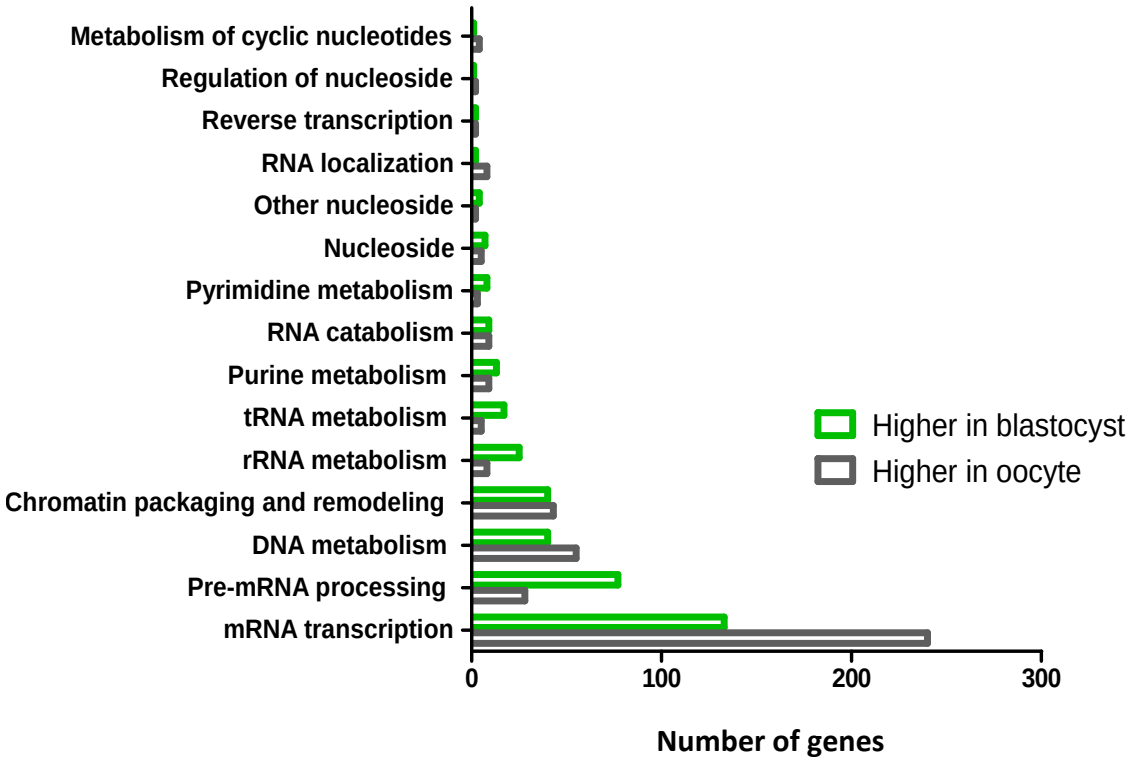

Figure 3. 40: Sub-categories of the nucleic acid metabolism biological process. The majority of genes involved in mRNA transcription showed higher expression in the oocyte. Genes involved in pre-mRNA processing, mostly in mRNA splicing, showed higher expression at the blastocyst stage. x axis: number of genes, y axis: PANTHER biological process

When this data were further limited to those probes that showed a 10-fold or higher level change, 1693 probes were identified. Of these, 722 (679 genes) were overexpressed ($\log_2$(ratio)>3.32) and 970 probes (918 genes) were underexpressed ($\log_2$(ratio) < -3.32) in the blastocyst compared to the oocyte.

The 10-fold change genes were analyzed based on molecular function. The majority of blastocyst upregulated genes belonged to the nucleic acid binding category (131 genes) and of these most were annotated “ribosomal proteins”, “other RNA-binding proteins”, “translation factors”, “histone” or “helicase” (figure 3.41).

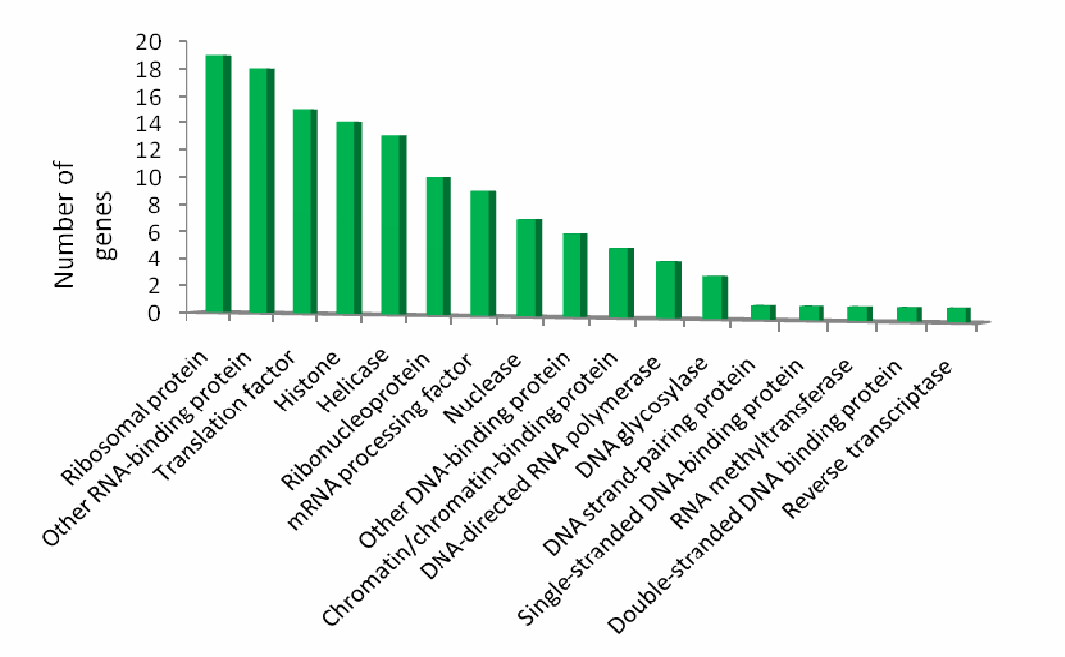

Figure 3. 41: Grouping of 131 nucleic acid binding category genes (128 function hits) that were 10-fold up-regulated in the human blastocyst.

The majority of blastocyst downregulated genes were also of “nucleic acid binding” molecular function (105 genes), and most were annotated “other DNA-binding protein”, “nuclease” and “chromatin/chromatin-binding protein” (figure 3.42). The second biggest category of downregulated genes was the “transcription factor” category, comprising 100 genes, 53% of them being “zinc-finger transcription factors”.

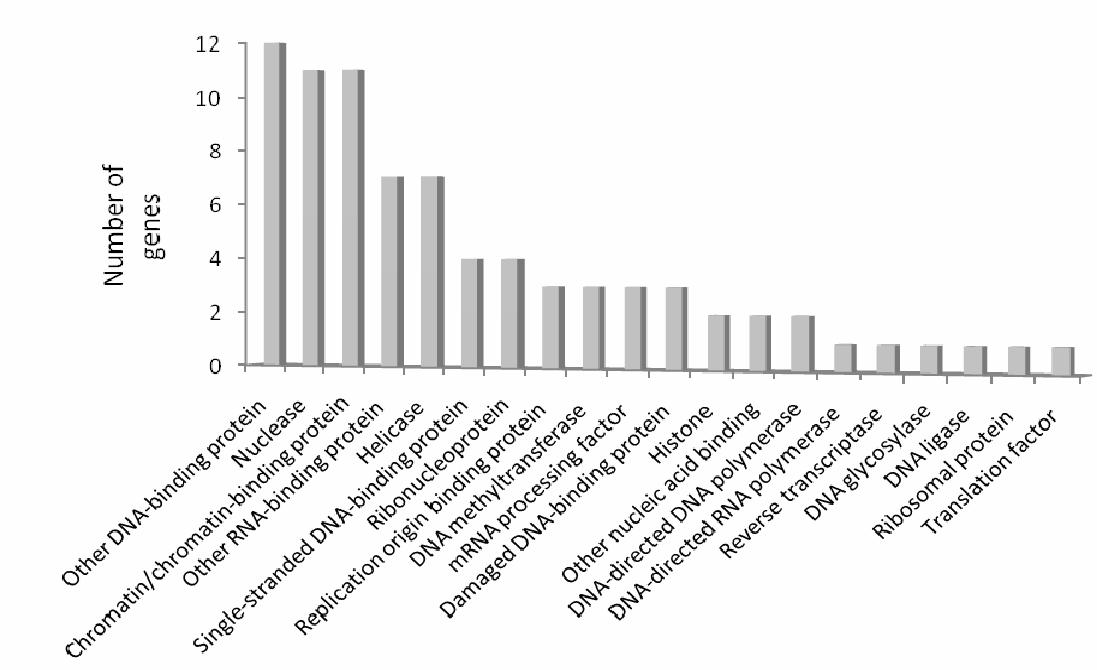

Figure 3. 42: Grouping of 105 nucleic acid binding genes (80 hits) 10-fold down-regulated in the blastocyst

3.3.2.5 Oocyte and blastocyst-specific genes

Genes expressed in oocytes only (maternal) or blastocysts only (embryonic) were identified. Gene search was performed using the Celera dataset gene ID (PANTHER database). There were 8772 genes with expression common to MII oocytes and blastocysts, 1909 genes were expressed in oocytes only and 3122 genes in blastocysts only (figure 3.43).

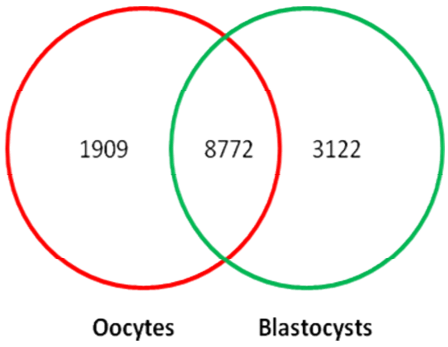

Figure 3. 43: Identification of genes unique in oocytes and blastocysts. Genes were identified by their Celera dataset ID.

The majority of genes uniquely expressed in oocytes, when grouped by biological process, were annotated unclassified (45.1%), signal transduction (14.1%), nucleic acid metabolism (12.3%), protein metabolism (10%) and developmental processes (9.8%). When grouped by molecular function most genes were either of unclassified function (43.7%), transcription factors (8%) or involved in nucleic acid binding (7.6%). Finally, when analyzed by PANTHER pathway, oocyte-specific genes mostly belonged to the following pathways: Wnt signaling pathway, Inflammation mediated by chemokine and cytokine signalling pathway, Huntington disease, Interleukin signaling pathway, PDGF signaling pathway, Angiogenesis, Integrin signaling pathway, TGF-beta signaling pathway, Cadherin signaling pathway, B cell activation and G-protein signaling pathway.

Similarly, blastocyst-specific genes, when grouped by biological process, were annotated unclassified (42.9%), nucleic acid metabolism (15%), protein metabolism (12.1%), signal transduction (10.6%) and developmental processes (8%). By molecular function 40.7% of genes were unclassified, 12.9% had a role in nucleic acid binding and 8.7% were transcription factors. Important pathways of blastocyst-specific genes were annotated in the following order: Wnt signaling pathway, Interleukin signaling pathway, integrin signalling pathway, inflammation mediated by chemokine and cytokine signalling pathway, angiogenesis, PDGF signaling pathway, TGF-beta signalling pathway, EGF receptor signaling, FGF signaling pathway and p53 pathway.

Therefore, the main differences in gene expression that were generally observed between oocytes and embryos were also reflected in the analysis of oocyte and blastocyst-specific gene expression. Overall, the Wnt signalling pathway was the most represented in both oocyte and blastocyst-specific genes. Other oocyte pathways, particularly the inflammation mediated by chemokine and cytokine signaling pathway showed expression of a similar number of genes as the Wnt signalling pathway.

In order to identify which genes are most significant at each developmental stage, the oocyte and blastocyst-specific genes were then separately grouped by expression level (as in table 3.24). There were 349 of the oocyte-specific genes and 431 of the blastocyst-specific genes that showed high expression levels listed in appendix 3 tables A3.3 and A3.4. Figures 3.44 and 3.45 allow a direct comparison of these, both by biological process and molecular function. On analysis by PANTHER pathway of high

expression genes, the main pathway for MII oocytes remained the Wnt signaling pathway, whereas the high expression blastocyst specific genes belonged to the integrin signaling pathway, cytoskeletal regulation by Rho GTPase, angiogenesis and PDGF signaling pathway. A summary of sample-specific gene expression, with a focus on highly expressed genes is given in tables 3.27 and 3.28.

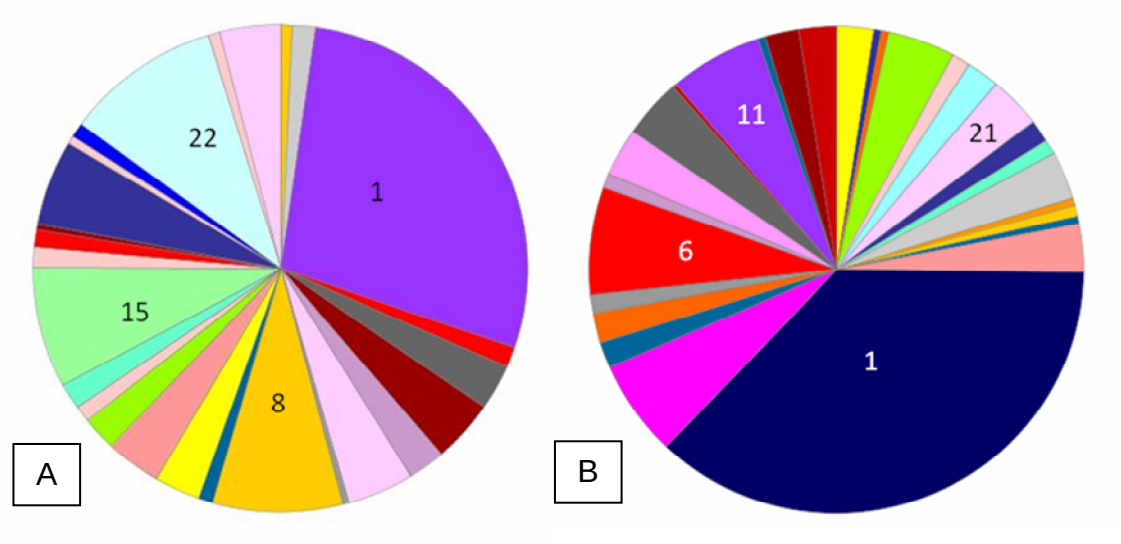

Figure 3. 44: Grouping by A) PANTHER Biological process and B) PANTHER Molecular function of 349 high expression genes unique in oocytes (categories numbered clock-wise). Percentage of gene hits per category against total number of genes is indicated in brackets. Categories not detected in the high expression blastocyst-specific genes are underlined. The five biggest categories are indicated in bold type.

A) **Biological process. 1: Unclassified (42.4%)** 2.Carbohydrate metabolism (2%). 3. Cell adhesion (4.6%). 4. Cell cycle (6.3%). 5. Cell proliferation and differentiation (3.7%). 6. Cell structure and motility (6.6%). 7. Coenzyme and prosthetic group metabolism (0.6%). **8. Developmental processes (12.9%)**. 9. Homeostasis (1.4%). 10. Immunity and defense (4.6%). 11. Intracellular protein trafficking (5.4%). 12. Lipid, fatty acid and steroid metabolism (3.4%). 13. Muscle contraction (1.7%). 14. Neuronal activities (2.6%). **15. Nucleoside, nucleotide and nucleic acid metabolism (12%)**. 16. Oncogenesis (2%). 17. Other metabolism (1.7%). 18. Phosphate metabolism (0.6%). **19. Protein metabolism and modification (8.6%)**. 20. Protein targeting and localization (0.9%). 21. Sensory perception (1.4%). **22. Signal transduction (15.5%)**. 23. Sulfur metabolism (1.1%). 24. Transport (6%). 25. Amino acid metabolism (1.1%). 26. Apoptosis (2.3%)

B) **Molecular function. 1. Unclassified (43.3%)**. **2. Nucleic acid binding (7.4%)**. 3. Oxidoreductase (2%). 4. Phosphatase (2.3%). 5. Protease (1.4%). **6. Receptor (8.3%)**. 7. Select calcium binding protein (1.1%). 8. Select regulatory molecule (3.7%). 9. Signaling molecule (4.6%). 10. Synthase and synthetase (0.3%). **11. Transcription factor (7.2%)**. 12. Transfer/carrier protein (0.6%). 13. Transferase (2.6%). 14. Transporter (2.9%). 15. Cell adhesion molecule (2.9%). 16. Cell junction protein (0.6%). 17. Chaperone (0.6%). **18. Cytoskeletal protein (5.2%)**. 19. Defense/immunity protein (1.4%). 20. Extracellular matrix (2.6%). 21. Hydrolase (4%). 22. Ion channel (1.7%). 23. Isomerase (1.1%). 24. Kinase (3.7%). 25. Ligase (0.6%). 26. Lyase (0.9%). 27. Membrane traffic protein (0.6%). 28. Miscellaneous function (3.7%).

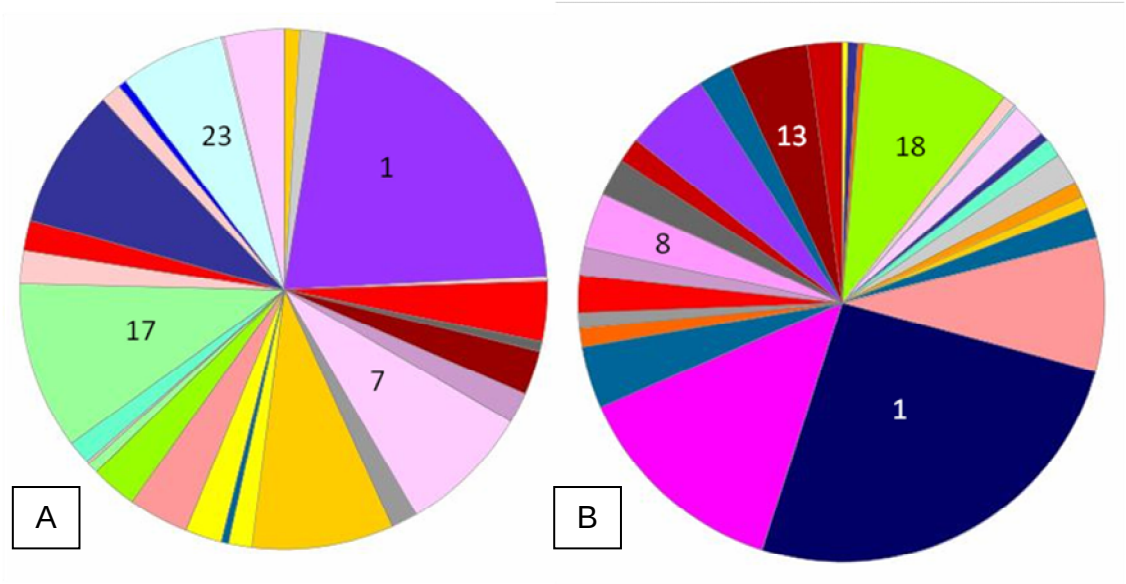

Figure 3. 45: Grouping by A) PANTHER Biological process and B) PANTHER Molecular function of 431 high expression genes unique in human blastocysts (categories numbered clock-wise).
Percentage of gene hit per category against total number of genes is indicated in brackets. Categories not detected in high expression oocyte-specific genes are underlined. The five biggest categories are indicated in bold type.

A) Biological process. 1: Unclassified (31.3%) 2.Blood circulation and has exchange (0.5%). 3. Carbohydrate metabolism (5.3%). 4. Cell adhesion (0.9%). 5. Cell cycle (3.9%). 6. Cell proliferation and differentiation (2.8%). 7. Cell structure and motility (11.8%). 8. Coenzyme and prosthetic group metabolism (2.3%). **9. Developmental processes (12.5%)**. 10. Electron transport (2.1%). 11. Homeostasis (0.7%). 12. Immunity and defense (3.2%). 13. Intracellular protein trafficking (5.3%). 14. Lipid, fatty acid and steroid metabolism (4.2%). 15. Miscellaneous (0.9%). 16. Neuronal activities (2.1%). 17. **Nucleoside, nucleotide and nucleic acid metabolism (15.1%)**. 18. Oncogenesis (3%). 19. Other metabolism (2.8%). **20. Protein metabolism and modification (12.5%)**. 21. Protein targeting and localization (1.9%). 22. Sensory perception (0.7%). **23. Signal transduction (9.3%)**. 24. Sulfur metabolism (0.2%). 25. Transport (5.3%). 26. Amino acid metabolism (1.4%). 27. Apoptosis (2.3%).

B) Molecular function. 1. Unclassified (30.9%). **2. Nucleic acid binding (16.2%)**. 3. Oxidoreductase (4.6%). 4. Phosphatase (1.4%). 5. Protease (1.2%). 6. Receptor (2.8%). 7. Select calcium binding protein (2.1%). 8. Select regulatory molecule (4.2%). 9. Signaling molecule (2.8%). 10. Synthase and synthetase (1.9%). **11. Transcription factor (6.3%).** 12. Transfer/carrier protein (2.6%). 13. Transferase (5.8%). 14. Transporter (2.6%). 15. Cell adhesion molecule (0.5%). 16. Cell junction protein (0.7%). 17. Chaperone (0.5%). **18. Cytoskeletal protein (11.1%).** 19. Defense/immunity protein (0.9%). 20. Extracellular matrix (0.2%). 21. Hydrolase (2.6%). 22. Ion channel (0.7%). 23. Isomerase (1.4%). 24. Kinase (2.3%). 25. Ligase (1.2%). 26. Lyase (0.9%). 27. Membrane traffic protein (2.3%). **28. Miscellaneous function (10%).**

Table 3. 27: Grouping of oocyte and blastocyst-specific gene categories by PANTHER biological process. Bold type indicates points of discussion.

Biological Process	Main represented gene categories			
	Oocyte-specific genes	**High expression oocyte-specific genes**	**Blastocyst-specific genes**	**High expression blastocyst-specific genes**
Carbohydrate metabolism	Other polysaccharide metabolism	**Glycogen metabolism, glycolysis, other**	Other polysaccharide metabolism	**Glycolysis**
Cell cycle	Cell cycle control	Cell cycle control, mitosis (chromosome segregation)	Cell cycle control, mitosis	Mitosis (chromosome segregation)
Cell structure and motility	Cell structure	Cell motility, cell structure	Cell structure	Cell structure
Coenzyme and prosthetic group metabolism	Vitamin/cofactor transport	Vitamin/cofactor transport, other	Coenzyme metabolism, Vitamin/cofactor transport	Porphyrin metabolism, vitamin metabolism
Developmental processes	Ectoderm development	Ectoderm development	Ectoderm development	Ectoderm development
Electron transport	Other	n/a	Oxidative phosphorylation	**Oxidative phosphorylation**
Homeostasis	Calcium ion homeostasis, other	**Calcium ion, growth factor homeostasis**	Other	Other
Immunity and defense	Stress response	Stress response, blood clotting	Stress response	Blood clotting, detoxification,macrophage mediated immunity, stress response
Intracellular protein trafficking	Endocytosis	Endocytosis, exocytosis	Endocytosis (general vesicle transport)	General vesicle transport
Lipid, fatty acid and steroid metabolism	Lipid metabolism	**Lipid, phospholipid metabolism**	Steroid metabolism (cholesterol)	**Steroid metabolism (cholesterol)**
Neuronal activities	Synaptic transmission	Synaptic transmission	Synaptic transmission	Other
Nucleoside, nucleotide and nucleic acid metabolism	mRNA transcription (regulation)	mRNA transcription (regulation)	mRNA transcription (regulation)	mRNA transcription (regulation)
Oncogenesis	Tumor suppressor	Oncogene, tumor suppressor	Other	Tumor suppressor, other
Protein metabolism and modification	Protein modification	Protein modification (phosphorylation)	Protein modification (phosphorylation)	Protein biosynthesis, protein modification (phosphorylation)
Protein targeting and localization	Protein targeting	Asymmetric protein localization	Protein targeting	Protein targeting
Signal transduction	Cell surface receptor mediated signal transduction	**Cell surface receptor mediated signal transduction (G-protein mediated)**	Cell surface receptor mediated signal transduction (G-protein)	**Cell communication (cell-adhesion mediated signaling)**
Transport	Ion transport (cation)	Ion transport (cation)	Ion transport (cation)	Ion transport (cation)
Amino acid metabolism	Amino acid transport	Other metabolism	Amino acid catabolism	Amino acid biosynthesis, catabolism
Apoptosis	Inhibition of apoptosis	Inhibition of apoptosis	Induction of apoptosis	Inhibition of apoptosis

Table 3. 28: Grouping of oocyte and blastocyst-specific gene categories by PANTHER molecular function. Bold type indicates points of discussion

Molecular Function	Main represented gene categories			
	Oocyte-specific genes	**High expression oocyte-specific genes**	**Blastocyst-specific genes**	**High expression blastocyst-specific genes**
Nucleic acid binding	Other RNA-binding protein	**Chromatin/chromatin-binding protein**	Ribosomal protein	**Other RNA-binding protein**
Oxidoreductase	Dehydrogenase	Dehydrogenase, other oxidoreductase	Dehydrogenase	Dehydrogenase
Phosphatase	Protein phosphatase	Protein phosphatase	Protein phosphatase	Other phosphatase
Protease	Serine protease	Serine protease	Serine protease	Metalloprotease
Receptor	Other receptor (G-protein)	**Other receptor**	G-protein coupled receptor	Other receptor
Select calcium binding protein	Calmodulin related protein	Calmodulin related protein	Calmodulin related protein	Calmodulin related protein
Select regulatory molecule	G-protein modulator	G-protein, kinase modulator	G-protein modulator (G-protein)	G-protein, kinase modulator
Signaling molecule	Other signaling molecule	Growth factor	Other signaling molecule	Other signaling molecule
Synthase and synthetase	Synthase and synthetase	Synthase (100%)	Synthase	Synthetase
Transcription factor	Zinc finger transcription factor	Zinc finger transcription factor	Zinc finger transcription factor	Zinc finger transcription factor
Transfer/carrier protein	Other transfer/carrier protein	**Mitochondrial carrier protein**	Other transfer/carrier protein	**Other transfer/carrier protein**
Transferase	Glycosyltransferase	**Acyltransferase**	Methyltransferase	**Methyltransferase**
Transporter	Other transporter	Other transporter	Other transporter	Other transporter
Cell adhesion molecule	Other cell adhesion molecule	Other cell adhesion molecule	Other cell adhesion molecule	Other cell adhesion molecule
Cell junction protein	Tight junction, other	**Tight junction**	Tight junction	**Gap junction**
Chaperone	Other chaperones	Other chaperones	Other chaperones	Chaperonin
Cytoskeletal protein	Microtubule family cytoskeletal protein	**Microtubule family cytoskeletal protein**	Intermediate filament	**Intermediate filament**
Defense/immunity protein	Immunoglobulin, other	Complement component	Immunoglobulin receptor family member	Antibacterial response protein, complement component, other
Extracellular matrix	Extracellular matrix glycoprotein	Extracellular matrix glycoprotein	Extracellular matrix glycoprotein	Extracellular matrix linker protein
Hydrolase	Lipase, other hydrolase	Other hydrolase	Other hydrolase	Other hydrolase
Ion channel	Voltage-gated ion channel	Voltage-gated ion channel	Voltage-gated ion channel	Voltage-gated ion channel
Isomerase	Epimerase/racemase	Other isomerase	Epimerase/racemase, other isomerase	Other isomerase
Kinase	Protein kinase	Protein kinase	Protein kinase	Protein kinase
Ligase	Ubiquitin-protein ligase	Ubiquitin-protein ligase	Ubiquitin-protein ligase	Other ligase, Ubiquitin-protein ligase
Lyase	Cyclase	**Decarboxylase**	Dehydratase	Dehydratase
Membrane traffic protein	Membrane traffic regulatory protein	Membrane traffic regulatory protein, SNARE protein	Membrane traffic regulatory protein	Membrane traffic regulatory protein
Miscellaneous function	Other	Other	Structural protein	Structural protein

3.3.3 Investigation of potential housekeeping gene expression

Housekeeping genes (HKG) investigated in human oocytes and embryos included:

- Genes generally involved in maintaining basic cell functions
- Genes common in three published datasets investigating HK gene expression on differentiated adult tissue, and
- Genes stably expressed in undifferentiated and differentiating human embryonic stem cells (hESCs)

3.3.3.1 Genes maintaining basic cell functions

The expression of 411 housekeeping genes involved in transcription, transport, translation and proteolysis was investigated in MII oocytes and blastocyst stage embryos, with a focus on those genes with a significantly different expression between the two samples or higher than 10 times fold change (411 genes reported by Zhu et al., 2008).

For 164 genes the detected signal value showed a statistically significant difference between oocytes and blastocysts. Most genes (119) were up-regulated in the blastocyst, while 45 of them were down-regulated. Although most categories indicated both up- and down-regulated genes, in certain groups, tRNA synthetase, hnRNP and snRNP, all genes with a significant difference were found up-regulated in the blastocyst (table 3.29).

In contrast, genes belonging to the nuclear pore complex category were found down-regulated in the blastocyst sample. The genes with over a 10-fold positive or negative difference in expression between the two samples are also indicated in table 3.29. The *SFRS2* gene, involved in mRNA splicing, is highlighted (table 3.29) for being over 100 times upregulated in the blastocyst sample.

3.3.3.2 Genes identified as HKG from adult tissue studies

Further analysis involved other genes commonly considered as housekeeping genes based on information from three previously published datasets investigating HKG in differentiated adult tissue. Although each dataset provided a list of approximately 500 genes, only 155 were shared between them. The current investigation focused on these common genes (list kindly provided by Dr. Zhu, personal communication), excluding those, mainly ribosomal proteins, that had already being included in the previous search of 411 genes. A total of 125 genes were investigated. Table 3.30 summarizes results. As previously, the >10-fold up and down-regulated genes are indicated. Similarly, the majority of genes with differential expression were up-regulated in the blastocyst sample. Commonly used genes, *ACTB* and *GAPDH*, showed over a 10-fold difference in expression between the two samples.

Table 3. 29: Investigation of 411 HKGs in human MII oocytes and blastocyst stage embryos. Each column indicates the number of probe ID and/or number of genes detected. The human oocyte was used as the control. Genes up-regulated and down-regulated in the human blastocyst are indicated here. +: up-regulated genes, -: down-regulated genes, FC: fold change

Gene category (number of genes)	**Oocytes** (number of probe ID hits/ number of genes detected)	**Blastocysts** (number of probe ID hits/ number of genes detected)	**p<0.05** (probe ID/ genes detected) (number of genes +/-)	**FC >10** (gene symbol) +	-
Transcription pre-initiation complex (40)	32/31	32/30	14/14 (5+/9-)	n/a	*CDK7, GTF2B*
Transcription elongation complex (17)	15/15	17/15	12/10 (7+/3-)	*SUPT4H1, TCEB2, RDBP*	*CCNT1*
Essential splicing factor (31)	29/24	33/26	13/12 (11+/1-)	***SFRS2***, *SFRS3, YBX1*	n/a
hnRNP (18)	18/16	21/17	9/9 (9+)	*HNRPA3*	n/a
snRNP (32)	29/29	31/31	12/12 (12+)	n/a	n/a
Capping related genes (5)	4/3	5/4	1/1 (1+)	n/a	n/a
Cleavage and polyadenylation complex (13)	12/10	13/11	6/6 (4+/2-)	*FLJ12529, COLEC12*	n/a
Nuclear Pore complex (28)	25/23	25/23	12/11 (11-)	n/a	*NUP35, NUP133*
Translation initiation, elongation and termination factor (37)	34/31	40/36	24/22 (20+/2-)	*ETF1, E1F5, E1F4A1, E1F3S1, E1F4EBP1*	n/a
tRNA synthetase (20)	17/17	19/19	12/12 (12+)	*AARS, WARS*	n/a
Cytosolic ribosome (82)	102/68	103/71	31/26 (24+/2-)	*MRPS12, RPL34*	n/a
Ubiquitin mediated proteolysis (45)	34/33	32/31	15/14 (4+/10-)	*CDC23, TCEB2*	*HERC1, SKP2, BTRC, SKP1A*
Proteasome (43)	31/31	37/35	15/15 (10+/5-)	*PSMC3, PSME2*	n/a
Total: 411	382/331	408/349	176/164 (119+/45-)		

Table 3. 30: Investigation of 125 HKGs genes, common in three HKG datasets, in human oocytes and blastocyst stage embryos. +: upregulated genes, -: downregulated genes, FC: fold change

Gene category (number of genes)	**Oocytes** (number of probe ID hits/ number of genes detected)	**Blastocysts** (number of probe ID hits/ number of genes detected)	**p<0.05** (probe ID/ genes detected) (number of genes +/-)	**FC >10** (gene symbol) +	-
125 other common HK genes	105/96	115/103	52/47 (36+/11-)	***ACTB***, *ALDOA*, *ATF4*, *ENO1*, ***GAPDH***, *GPX4*, *MRPS12*, *MYL6*, *NCL*, *NONO*, *RPL34*, *TMSB10*, *TUBB2A*	*FKBP1A* *USP11*

Overall, out of the 536 HKGs investigated (411+125 genes, sections 3.3.3.1 and 3.3.3.2), 427 were detected in the oocyte and 452 in the blastocyst. For 277 of HKGs detected in the oocytes (64.87%), and 355 of the blastocyst genes (78.5%), the probe signal detected on analysis was high. 90.9% of the high signal oocyte HKGs (252 genes) remained of high signal in the blastocyst samples. The genes that maintained a high signal in both oocytes and blastocysts are listed in table A3.5 of appendix 3.

Figure 3.46 indicates the expression levels in oocytes and blastocysts of several of the commonly used housekeeping genes and figure 3.47 lists genes found in other studies to be stably expressed during preimplantation development of the human and other species' embryos, and the corresponding results from the analysis of human oocytes and blastocysts in this study. *B2M* (a major histocompatibility complex antigen), *HIST1H2AA* and *HIST3H2A* (histones) were only detected at the blastocyst stage.

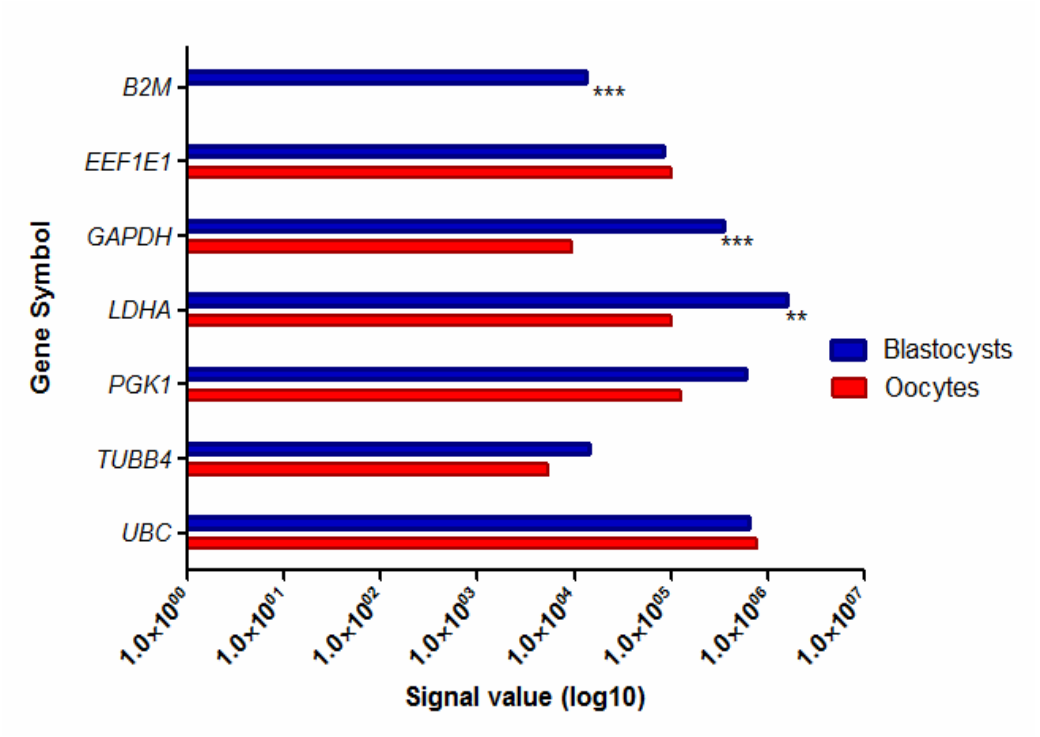

Figure 3. 46: Expression levels of selected commonly used HKGs in the human oocyte and blastocyst. **p<0.01, ***p<0.001

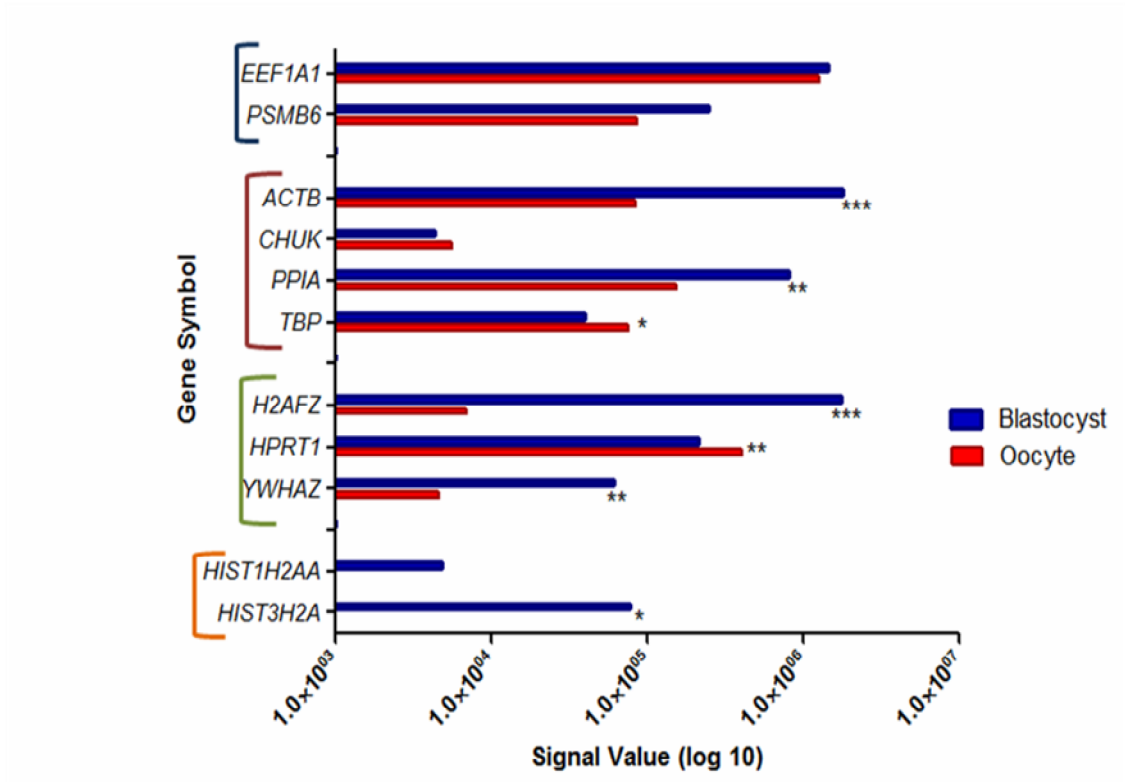

Figure 3. 47: Signal values of genes found to be stably expressed in different species. Blue brackets: human. Red brackets: mouse. Green brackets: rabbit. Orange brackets: bovine.
*p<0.05, **p<0.01, ***p<0.001

All detected genes in the parametric comparison were listed based on their differences in expression between the two samples. The smallest differences in expression levels were observed for the following 20 genes, most of which are involved in protein and nucleic acid metabolism biological processes (table 3.31).

Table 3. 31: Genes showing similar expression levels between the human MII oocytes and blastocysts

Gene Symbol	Gene Name
CTNNB1	catenin (cadherin-associated protein), beta 1, 88kDa
EEF1D	eukaryotic translation elongation factor 1 delta (guanine nucleotide exchange protein)
ASCC3L1	activating signal cointegrator 1 complex subunit 3-like 1
WDR57	WD repeat domain 57 (U5 snRNP specific)
PCBP2	poly(rC) binding protein 2
RPL23A	ribosomal protein L23a
GTF2A2	general transcription factor IIA, 2, 12kDa
SFRS5	splicing factor, arginine/serine-rich 5
RPL28	ribosomal protein L28
POLR2F	polymerase (RNA) II (DNA directed) polypeptide F
CLN5	ceroid-lipofuscinosis, neuronal 5
RPL37A	ribosomal protein L37a
TCEB1	transcription elongation factor B (SIII), polypeptide 1 (15kDa, elongin C)
PFDN5	prefoldin 5
CARS	cysteinyl-tRNA synthetase
EIF3S5	eukaryotic translation initiation factor 3, subunit 5 epsilon, 47kDa
SNRPD3	small nuclear ribonucleoprotein D3 polypeptide 18kDa
PTDSS1	phosphatidylserine synthase 1
RPS23	ribosomal protein S23
ARF1	ADP-ribosylation factor 1

The first twenty signals of highest intensity out of all investigated genes in both oocytes and blastocysts are shown in table 3.32 in order of expression from high to low. Amongst these, eight genes were common between the two samples.

Table 3. 32: List of twenty highest signal probes detected in the human MII oocyte and embryo blastocyst. Bold type highlights genes present in both sample lists.

Oocyte		Blastocyst	
Gene Symbol	**Gene Name**	**Gene Symbol**	**Gene Name**
RPLP1	**ribosomal protein, large, P1**	*RPS4X*	ribosomal protein S4, X-linked
RPLP1	**ribosomal protein, large, P1**	***RPS11***	**ribosomal protein S11**
RPL7A	**ribosomal protein L7a**	***RPS11***	**ribosomal protein S11**
H3F3B	H3 histone, family 3B (H3.3B)	***RPLP1***	**ribosomal protein, large, P1**
UBB	ubiquitin B	***RPLP1***	**ribosomal protein, large, P1**
SKP2	S-phase kinase-associated protein 2 (p45)	*PABPC1*	poly(A) binding protein, cytoplasmic 1
CDK7	cyclin-dependent kinase 7 (MO15 homolog, Xenopus laevis, cdk-activating kinase)	*PRDX1*	peroxiredoxin 1
EEF1A1	eukaryotic translation elongation factor 1 alpha 1	*RPL24*	ribosomal protein L24
RPS12	ribosomal protein S12	*ENO1*	enolase 1, (alpha)
RPS12	ribosomal protein S12	*RPS27A*	ribosomal protein S27a
RPS11	**ribosomal protein S11**	*RPS27A*	ribosomal protein S27a
RPS11	**ribosomal protein S11**	***EEF2***	**eukaryotic translation elongation factor 2**
RPL41	ribosomal protein L41	***EEF2***	**eukaryotic translation elongation factor 2**
EEF1A1	eukaryotic translation elongation factor 1 alpha 1	*RPS7*	ribosomal protein S7
SNRPE|SNRPEL1	small nuclear ribonucleoprotein polypeptide E|small nuclear ribonucleoprotein polypeptide E-like 1	***RPLP2***	**ribosomal protein, large, P2**
RPLP2	**ribosomal protein, large, P2**	*RPL18*	ribosomal protein L18
RPL41	ribosomal protein L41	*RPL18*	ribosomal protein L18
RPS23	ribosomal protein S23	*RPL5*	ribosomal protein L5
EEF2	**eukaryotic translation elongation factor 2**	***RPL7A***	**ribosomal protein L7a**
EEF2	**eukaryotic translation elongation factor 2**	*ACTB*	actin, beta

3.3.3.3 Genes identified as HKG from undifferentiated and differentiating stem cell lines

Synnergren et al., (2007), identified the 24 most stably expressed genes in undifferentiated and early differentiating hESCs and the expression of these was investigated in our oocyte and embryo samples. Thirteen out of these 24 genes were detected (table 3.33). For four of them, underlined in the table, there was a significant difference (adj. $p<0.05$) in the signal detected between the two samples. *FLJ20186* and

HDDC2, both of uncharacterized molecular function, were over 10-fold upregulated in the blastocyst sample.

Table 3. 33: Level of expression in the human oocyte and blastocyst for 13 of the 24 most stably expressed genes in differentiating hESCs. Underlined gene symbols indicate a statistically significant difference. The human MII oocyte was used as the control. +FC: up-regulated in the blastocyst, compared to the MII oocyte, -FC:down-regulated in the blastocyst, compared to the MII oocyte. Bold type indicates genes that were >10-fold up-regulated.ND: no statistically significant difference observed

			Expression level		
Gene ID (Celera ID provided unless otherwise indicated)	**Gene Symbol**	**Gene Name**	**Oocyte**	**Blastocyst**	**Parametric comparison (adj.p value)**
hCG32850	*CDC14A*	CDC14 cell division cycle 14 homolog A			
hCG36828	*KIAA0141*	KIAA0141			
hCG1811676	***FLJ20186***	hypothetical protein FLJ20186	Low	High	<0.01,+FC
hCG1983988	*RPL7*	ribosomal protein L7			
hCG14859	*NUBP1*	nucleotide binding protein 1	Medium	High	ND
hCG1646292	*FLJ33977*	DTW domain containing 2			
hCG19988	***HDDC2***	HD domain containing 2	Medium	High	<0.01,+FC
hCG41427	*PLEKHA1*	pleckstrin homology domain containing, family A	High	High	<0.05,-FC
hCG40180	*RIPK3*	receptor-interacting serine-threonine kinase 3	High	Medium	ND
hCG1999395	*RND1*	Rho family GTPase 1	Medium	Low	<0.01, -FC
hCG23905	*CPNE2*	copine II			
hCG1810870	*POLE*	polymerase (DNA directed), epsilon			
hCG32054	*VSNL1*	visinin-like 1			
hCG2033817	*GTF2H3*	general transcription factor IIH, polypeptide 3, 34kDa			
hCG18037	*ELN*	elastin	n/a	Low	ND
Entrez: 5728	*PTEN*	phosphatase and tensin homolog	High	High	ND
hCG22397	*CA6*	carbonic anhydrase VI			
hCG23039	*SLC5A11*	solute carrier family 5 (sodium/glucose cotransporter), member 11	High	n/a	
hCG20493	*MLH3*	mutL homolog 3			
hCG15226	*STIM1*	stromal interaction molecule 1	Medium	Medium	ND
hCG16633	*CREBBP*	CREB binding protein	n/a	Medium	ND
hCG37392	*HM13*	histocompatibility (minor) 13	Medium	Medium	ND
hCG32870	*FOXP4*	forkhead box P4			
Entrez:440026	*TMEM41B*	transmembrane protein 41B	Low	Medium	ND

3.3.4 Assessment of gene expression for important functional pathways

Further analysis of the gene expression data focused on the investigation of pathways associated with the disease mechanism in DM1 to identify genes present in oocytes and embryos that may be affected by the *DMPK* expansion. Current investigation involved genes regulating the microRNA processing pathway and methylation.

3.3.4.1 Investigation of genes involved in the microRNA processing pathway

Genes involved in microRNA processing were investigated in the oocyte and embryo samples (Mtango et al., 2008). Out of the 25 genes examined, 16 were detected in the oocyte and 17 in the embryo blastocyst sample (table 3.34, figure 3.48).

The investigation of the microRNA processing genes indicated that genes involved in RNA catabolism, *RNASEN* and *DICER1* are expressed in both oocytes and blastocysts, with *DICER1* showing significantly higher expression at the oocyte stage. High oocyte activity was also observed for genes involved in mRNA splicing.

GEMIN5, *GEMIN2* involved in mRNA splicing, and *TNRC6B* of the RISC complex showed higher expression levels in the oocyte. *YBX1*, *GEMIN4* and *MOV10*, with roles in regulation of transcription and translation, as well as the exportins *XPO1*, *XPO4* and *XPO5*, were significantly upregulated in the blastocyst.

Table 3. 34: Expression levels of genes involved in the microRNA processing pathway in the human MII oocyte and blastocyst. +FC: up-regulated in the blastocyst, -FC:down-regulated in the blastocyst, ND: no statistically significant difference observed

			Expression level		
Function	**Celera Gene ID**	**Gene Symbol**	**Oocytes**	**Blastocysts**	**Parametric comparison**
Microprocessing	hCG2003777	*DROSHA/ RNASEN*	High	High	ND
	hCG2001933	*DGCR8*	n/a	Low	ND
	hCG24439	*DICER1*	Medium	Medium	P<0.05, -FC
Exportins	hCG1986857	*XPO1*	Medium	High	P<0.05, +FC
	hCG1774337	*XPO4*	n/a	Medium	P<0.05, +FC
	hCG19013	*XPO5*	Low	Medium	P<0.05, +FC
	hCG41537	*RANGAP1*	n/a	Low	ND
	hCG18633	*FMR1*	Medium	Medium	ND
RNA editing	hCG18813	*ADAR*	Medium	n/a	ND
	hCG401275	*ADARB1*	Low	n/a	ND
mRNA splicing	hCG21235	*SIP1 (GEMIN2)*	Medium	Medium	P<0.05, -FC
	hCG27817	*GEMIN4*	Low	Medium	P<0.05, +FC
	hCG1979691	***GEMIN5***	High	Low	**P<0.01, >100-FC**
	hCG1818032	*GEMIN6*	High	High	ND
	hCG22150	*GEMIN7*	n/a	n/a	n/a
	4904	***YBX1***	High	High	**P<0.01, >10+FC**
RISC complex	hCG38282	*AGO1/EIF2C1*	n/a	n/a	n/a
	hCG18139	*AGO2/EIF2C2*	High	High	n/a
	hCG1640521	*EIF2C3*	n/a	n/a	n/a
	hCG1787714	*EIF2C4*	Low	Low	ND
	hCG33381	*RISC/SCPEP1*	n/a	Medium	n/a
	hCG41115	*TNRC6B*	Low	n/a	P<0.05, -FC
	hCG38463	*MOV10*	Low	Medium	P<0.05, +FC
	hCG22573	*HIWI/PIWIL1*	n/a	n/a	n/a
	hCG1818433	*MILI/PIWIL2*	n/a	n/a	n/a

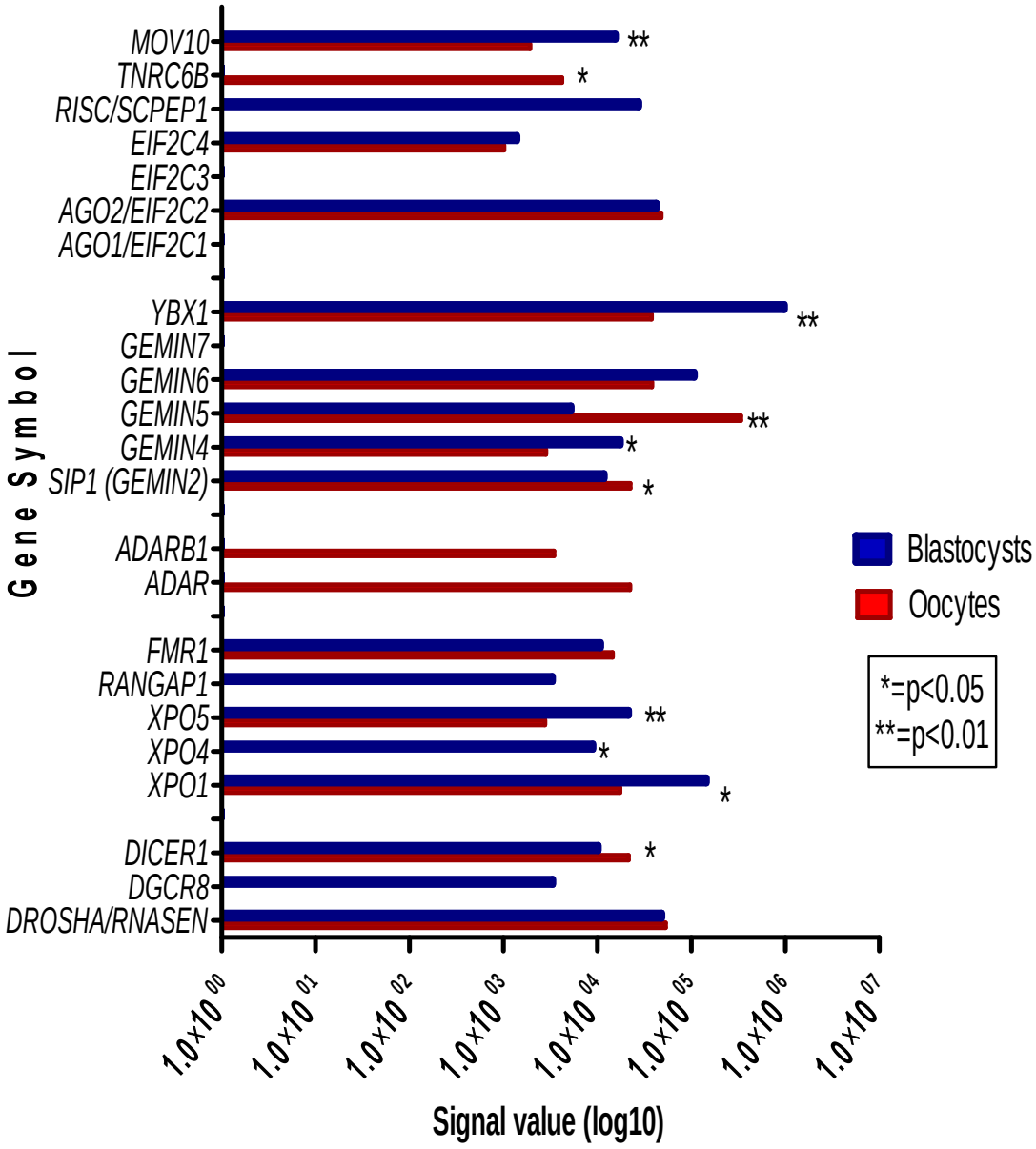

Figure 3. 48: Expression levels of microRNA machinery genes in human oocytes and embryo blastocysts.

3.3.4.2 DNA Methyltransferases in human oocytes and embryo blastocysts

Eighteen enzymes that methylate DNA bases were identified in the PANTHER database, based on their Celera ID and their expression was investigated. Nine of these were present in both oocytes and embryos, showing a medium and high expression level. *DNMT3L* and *METTL4* were detected at the blastocyst stage only. *DNMT1*, *HSMPP8*, *MGMT* and *POLS* showed higher expression in the oocyte, while *C21orf127*, *METTL4* and *RG9MTD1* gave higher expression signals for the blastocyst sample (table 3.35). Genes that were not detected included: *DNMT2*, *DNMT3A*, *KIAA1935*, *PWWP2*, *RG9MTD2* and *RG9MTD3*.

Table 3. 35: Expression levels of eleven human DNA methyltransferases (Celera database) present in the human MII oocyte and/or embryo blastocyst. +FC: upregulated in the blastocyst, -FC:downregulated in the blastocyst, ND: no statistically significant difference observed

		Expression level		
Celera Gene ID	**Gene Symbol**	**Oocytes**	**Blastocysts**	**Parametric Comparison**
hCG38847	*AOF2*	High	High	
hCG401157	*C21orf127*	Medium	High	P<0.05, +FC
hCG28474	*DNMT1*	High	High	**P<0.01, >10 -FC**
hCG37138	*DNMT3B*	Medium	High	ND
hCG401308	*DNMT3L*	n/a	High	n/a
hCG1774305	*HSMPP8*	Medium	Low	**P<0.01, >10 -FC**
hCG38411	*METTL4*	n/a	Low	P<0.05, +FC
hCG39601	*MGMT*	High	Medium	P<0.05, -FC
hCG24891	*PAPD5*	Medium	Low	ND
hCG18366	*POLS*	High	Medium	**P<0.01, >10 -FC**
hCG39275	*RG9MTD1*	Medium	High	P<0.05, +FC

3.3.4.3 Other DM1-related genes

Genes with a previously identified role in the DM1 pathogenesis include several transcription factors, DNA and RNA-binding proteins, mRNA processing and translation factors, ribonucleoproteins and genes implicated in DNA repair. Genes from these categories that have shown altered expression in normal vs. affected adult tissue experiments were investigated here.

MSH2, *MSH3*, *CUGBP1* and *SP3* factor showed significantly higher expression in the MII oocyte, while *MBNL3* was the only identified factor significantly upregulated at the blastocyst stage. Other important DM1-related genes, for example, *DMPK*, *MBNL1*, *MBNL2*, *PMS2* or *SP1* were not detected (table 3.36).

Table 3. 36: Expression levels of DM1-related genes (Celera database) in the human MII oocyte and embryo blastocyst. +FC: upregulated in the blastocyst, -FC:downregulated in the blastocyst, ND: no statistically significant difference observed

			Expression level		
Molecular Function (process)	**Gene ID**	**Gene Symbol**	**Oocytes**	**Blastocysts**	**Parametric comparison**
DNA-binding protein (DNA repair)	hCG17836	*MSH2*	High	High	P<0.01, -FC
	hCG37123	*MSH3*	Medium	Low	P<0.05, -FC
	hCG2008664	*PMS2*			
	hCG28368	*RAR,RAB40B*			
RNA-binding protein (mRNA splicing)	hCG15683	*PABPC1*	High	High	ND
	hCG25183	*CUGBP1*	Medium	Low	P<0.01, -FC
	hCG41209	*hnRNP,HNRPC*	High	High	ND
	hCG22986	*hnRNP,HNRPDL*	n/a	Medium	ND
Transcription factor (mRNA transcription)	hCG25794	*STAT1*	Medium	Medium	ND
	hCG1746842	*REST*	n/a	Low	ND
	hCG2007196	*RAR,RARA*	Low	Low	ND
	hCG1813145	*STAT3*	Low	Medium	ND
	hCG1787889	*SP1*			
	hCG17424	*SP3*	Medium	Low	**P<0.01,>10 -FC**
	hCG1789927.2	*SIX5*	Medium	Medium	ND
Double stranded binding protein (muscle development)	hCG27557	*MBLL,MBNL2*			
	hCG28028	*MBNL1*			
	hCG14618	*MBNL3*	n/a	High	**P<0.01,>10 +FC**
Protein Kinase	hCG1996612	*DMPK*			
Translation elongation factor	hCG23415	*EF1A*			

5 µg hybridizations

A total of 13170 probe IDs were detected in the Ba unaffected sample and 13768 were detected in the DMa affected sample. For both of these, 5µg labeled RNA was hybridized on the microarrays. When these two samples were compared using the PANTHER database gene expression tools, there was a significant over-representation of the mRNA transcription category in the DM1 affected sample (more genes involved in mRNA transcription were detected compared to the unaffected sample). No other differences were observed between the two samples. The two samples with the low RNA yield both showed expression of some genes, such as *SP1*, that were not detected in the 10µg hybridizations.

Even though no further analysis can be performed from the samples of low RNA yield, due to the lack of replicates, the information obtained can also provide information to assist further experiments for validation of the results by real-time PCR.

3.3.5 Summary of findings for section 3.3: Expression work

- **Gene expression profiling of human oocytes and embryo blastocysts**
  - A higher proportion of genes belonging to the nucleic acid metabolism and protein metabolism categories, in comparison to the other biological process categories, was detected in both human MII oocytes and blastocysts.
  - 5243 genes showed statistically significant differences in expression between oocytes and blastocysts. Of these, 2591 were under-expressed and 2652 were over-expressed in the blastocyst compared to the oocyte.
  - Genes with higher expression in the blastocyst belonged to the 'protein metabolism and modification' class and were mostly involved in protein biosynthesis.
  - The majority of over 10-fold blastocyst up-regulated genes, when grouped by molecular function, included "ribosomal proteins", "other RNA-binding proteins" or "translation factors", while most 10-fold blastocyst down-regulated genes were "other DNA-binding proteins", "nuclease" and "chromatin binding" proteins.
  - There were 8772 genes common to MII oocytes and blastocysts, 1909 genes were present in oocytes only and 3122 genes as blastocyst only.

- **Investigation of potential housekeeping gene expression**
  - The majority of genes with a potential housekeeping role were detected in oocytes and blastocysts with high expression levels
  - 90.9% of the high signal oocyte candidate HKGs remained of high signal in the blastocyst
  - Some commonly used housekeeping genes, including some identified to be stable during preimplantation development in human and other species, showed significant differences in expression between the two stages. *B2M*, *HIST1H2AA* and *HIST3H2A* were only detected at the blastocyst stage. Other genes with most similar expression levels were identified.
  - The majority of genes indicating stable expression during development according to studies on hESCs, showed significantly different expression between oocytes and blastocysts.

- **Assessment of gene expression for important functional pathways**
    - Detection of genes involved in the microRNA pathway, methylation, and DM1-related genes were identified and their levels of expression characterised as high, medium or low.

4. Discussion

4.1 Protocol development for preimplantation genetic diagnosis for a single gene disorder

4.1.1 PGD protocol development: general discussion

Preliminary genetic analysis is the first step in protocol development for PGD and involves determining conditions for mutation detection on genomic DNA as well as searching for informative polymorphic markers. When a couple is uninformative for all linked markers tested, an informative unlinked marker may be multiplexed along with the mutation primers instead. In this study, mutation detection was performed by F-PCR analysis to detect differences in size of the *DMPK* repeat alleles. F-PCR enables a cheaper, faster and less labour-intensive diagnosis, compared to other techniques, such as SSCP, RFLP or mini-sequencing. The main aim in protocol development was to simultaneously maximise PCR efficiency and accuracy. A fast diagnostic protocol is generally preferred because of the limited time period in which the analysis has to be performed, since the growing embryos may be held in culture up to day 6. Longer diagnostic protocols, for example CGH, therefore require embryo cryopreservation, followed by freezing and transfer in a subsequent cycle, after completion of the diagnosis.

In one occasion during this study, however, the PGD protocol involved a ‘split PCR’ reaction, to improve protocol efficiency. In ‘split PCR’, an initial multiplex reaction is performed and aliquots from the first reaction product are re-amplified in subsequent individual amplification reactions for each locus. This method is generally applied in PGD for monogenic disorders as it reduces optimisation time, although it increases the diagnosis time by introducing more amplification reactions.

Another way to reduce optimisation time is by performing blastocyst biopsy, which involves removal and amplification of more than a single cell for diagnosis so that protocols do not need to be optimised at the single cell level (McArthur et al., 2008). Other problems, however, may be encountered with blastocyst-stage biopsy, such as the smaller number of embryos that reach the blastocyst stage, the reduced time available for embryo transfer or the requirements for freezing. Additionally, due to time limitation, re-biopsy and re-testing in case of inconsistencies is not possible.

Overall, ‘split-PCR’ or other long PGD protocols, are used for isolated referred cases of uncommon mutations in order to reduce optimisation time, while for groups of patients

with a common mutation, such as the patients with DM1 in this study, efforts were focused towards establishing a universal, rather than patient-specific, PGD protocol. This would reduce the time required for workup at the single-cell level prior to PGD and eventually increase the number of patients that could be treated in a certain period of time.

Careful assessment of amplification, allele dropout and contamination rates was performed during optimisation to allow assessment of the risk of misdiagnosis following results from a PGD case. Several measures were established as standard practice during clinical diagnosis. For example, single lymphocytic cells from both the affected and unaffected partner were freshly isolated prior to the relevant PGD case, and stored at -80°C to be amplified alongside the blastomeres, as an indicator of successful amplification during the actual diagnosis procedure and to allow comparison of amplification between the single parental lymphocytes and single embryo blastomeres. Moreover, two, rather than one, PCR-mix negative controls were obtained during setup. These were additional to the cell-negative controls for monitoring contamination (table 3.12). The first negative control was obtained following setup of the PCR master mix and prior to aliquoting into the cell tubes, while the second negative control was obtained at the end of all aliquoting. The lack of amplification from the first PCR negative provided evidence that the reagents used for the setup of the PCR reaction were clear of contamination, while detection of contamination in the second PCR blank, indicated that contamination had occurred during the aliquoting. Finally, all cells were kept in the same order as obtained during cell biopsy, up until the last PCR reaction and analysis of results, so as to reduce the likelihood of handling errors and generally facilitate with monitoring of the procedure.

Overall, optimisation at the single cell level required the isolation and testing of approximately 50-200 single cells, while each optimised PCR-PGD protocol was tested on a minimum of 50 single cells prior to clinical application. At the early stages of protocol development, human blastomeres were also used on two occasions to confirm the single cell amplification efficiencies and allele-dropout rates prior to the clinical application. The amplification results of a number of blastomeres isolated from the same embryo were combined to identify the expected embryo genotype (as parental alleles from the IVF patient donors were unknown). Because of the difficulties associated with obtaining the parental genotype from the blastomere analysis, but also

identifying true ADO or amplification failure and differentiating from a potential chromosomal abnormality, this practice was stopped.
The practice of optimisation at the single cell level changed considerably over time, regarding both the type of cell used and the method of cell lysis.

4.1.1.1 Impact of cell type

The impact of cell type on single cell amplification has been previously investigated. One study compared the amplification efficiencies from single blastomeres, fibroblasts and polar bodies, and indicated significantly higher allele dropout (ADO) rates in blastomeres (Rechitsky et al., 1998). In another study, blastomeres were associated with slightly lower, but not significantly different, amplification efficiency and ADO rates compared to buccal cells (Piyamongkol et al., 2003). In our analysis, amplification efficiency for the single blastomeres was significantly lower compared to results from single lymphocytes or buccal cells.
At the beginning of this study buccal cells were preferred for protocol optimisation, as they were more readily available and obtained in a less invasive way. When buccal cells, however, were compared with amplification from single lymphocytes, results indicated a lower amplification efficiency and higher ADO rate in the former. An example of this is seen in the optimised DM1 triplex protocol (protocol 3), where the ADO rate from fifty-five single lymphocytes was lower (0-3.64% for the three markers) compared to analysis from fifty-three buccal cells (3.78-11.3%) (table 3.4). This difference is important considering that the general guidelines for good PGD practice from the Preimplantation Genetic Diagnosis International Society (PGDIS) (2004) emphasize that ADO over 10% is the main cause of PGD misdiagnosis. The differences in ADO detected with the different cell types are possibly the result of differences in cell quality, as single lymphocytes were in most cases freshly extracted and isolated cells, whereas buccal cells were often collected by the patients and sent by post (Piyamongkol et al., 2003).

Most problems regarding the cell type were observed during initial optimisation of the TP-PCR protocol, which involved the use of single buccal cells. The TP-PCR results from 160 buccal cells were very poor, inconsistent and generally associated with very high amplification failure rates. When practice moved over to testing of single

lymphocytes, the results were markedly improved. This finding agrees with previously reported recommendations that DNA of high quality should be used as the template particularly for TP-PCR amplification (Falk et al., 2006).

As a result, lymphocyte isolation became the standard practice for development of PGD protocols in general, despite the more time-consuming cell separation from blood and the generally more difficult isolation of the considerably smaller in size cells (buccal cell diameter ~65μm vs. lymphocyte diameter ~7 μm) (Ziyyat et al., 1999;Paszkiewicz et al., 2008). We reserved the use of buccal cells for DNA extraction and single cell work only for cases where relative's DNA had to be obtained from very young children (such as during phase allele investigation), or during follow-up of babies born after PGD.

4.1.1.2 Investigation of cell lysis methods

The method of cell lysis has also been proven to play an important role on the actual PCR amplification. Early studies supported ALB lysis for extraction of DNA from single cells over other methods such as freeze-thawing and the distilled water lysis method. In another study ALB lysis was associated with allele-specific amplification failure, which was not noted as a problem with PK/SDS lysis (Gitlin et al., 1996;el Hashemite and Delhanty, 1997). PK/SDS and ALB lysis were also compared by Thornhill et al., (2001) who investigated amplification for three genes (*CFTR*, *LAMA3* and *PKP1*) at different chromosomal loci on single lymphocytes. Amplification efficiencies were found to be comparable between the two methods but for two out of the three loci tested there was a statistically significant difference in ADO rates, with ALB lysis giving much lower ADO. Finally, a more recent study compared amplification efficiencies and ADO rates between five different cell lysis methods (liquid nitrogen method, distilled water lysis, alkaline lysis, proteinase K/SDS lysis buffer and N-lauroylsarcosine salt solution), using single lymphocytes as a template. The highest amplification efficiency and lowest ADO was observed with alkaline lysis, followed by PK/SDS lysis (Kim et al., 2008).

Results from the current study are also favourable towards alkaline lysis. The change in the proteinase K enzyme activity over time, possibly due to freeze-thawing, was one of

the difficulties encountered. To confirm that the required PK activity was still attained, a PK titration step was performed and cells were isolated in different concentrations of PK/SDS lysis buffers. The lysed cells were amplified using an optimised single cell PCR protocol, to determine which PK concentration allowed reproducibility of the results (i.e. expected efficiency, ADO rates). This requirement for adjustments of the PK enzyme concentration over time was not compatible with complete protocol standardisation, which is important in clinical practice. Furthermore, single cell amplification efficiencies were generally lower with PK lysis, compared to ALB (table 3.4).

For all the above reasons, our practice moved towards ALB lysis. The initial set up was more labour-intensive as it required the preparation and testing of a number of stock solutions of wash buffer (DB), tricine, BSA and DTT aliquots, as well as NaOH and KOH. Following this, however, the ALB lysis protocol was faster, requiring a 15min lysis step compared to 75min with PK. The single cells were almost immediately lysed when placed into the ALB lysis buffer. In the earlier stages of the study, this was considered to be a disadvantage, as it did not allow viewing of the cell under the microscope after tubing. This practice is, however, only considered necessary while practicing the technique of single cell tubing and is no longer required once proficiency and confidence in the technique is attained. During the learning curve of the tubing technique, however, the use of PK lysis, and viewing of the cell in the tube, allowed confirmation that any subsequent failure of amplification was not due to loss of the cell during transfer in the tube, thus ensuring that protocol efficiency was accurately estimated. With ALB lysis, the volume of wash buffer (DB) placed in the tube along with the cell was also critical and in several cases a large amount of buffer transferred along with the cell was associated with amplification failure. This difficulty was also overcome with practice, by ensuring that the single cells were transferred into a small volume, of just 1-2µl of wash buffer.

4.1.2 PGD for DM1

The decision to have a family can be very difficult for the DM1-affected individuals due to the unpredictability in DM1 inheritance, and the consequent difficulties in assessing the impact of a DM1 diagnosis on future pregnancies, offspring and other family members (Salehi et al., 2007).

From our group of patients, the major motivator for a DM1-affected couple in pursuing PGD was the experience of having relatives affected with the condition (22/23 couples). Because DM1 is a dominantly inherited disorder, couples may be faced with a number of terminations (TOP) before achieving a normal pregnancy. Overall, eight couples had experienced an affected pregnancy, and six of them had experienced TOP one or more times. One of the couples who had a TOP also had an affected daughter, while two other couples had lost a congenitally affected child. Seven of the referred couples had infertility problems, though not all necessarily DM1 related.

The main aim of developing PGD for DM1 has been to ensure the availability of widely applicable and reliable PGD protocols that can minimise the workup time. In this way, in cases of infertility, where IVF treatment is required, the availability of readily applicable PGD protocols would render PGD simply an additional step to the standard IVF procedure.

The emotional difficulties of the IVF/PGD procedure were underlined in this study by the two couples producing only two embryos following IVF. In both of these cases, the couples were adamant that they did not wish to proceed with the treatment. They found the procedure extremely stressful and with only two embryos available for testing they felt the chances of detecting and transferring an unaffected embryo and hopefully establishing an unaffected pregnancy were very low.

The emotional, along with the physical burden of the procedure but also the considerable cost of the IVF/PGD treatment (to cover fertility checks, IVF cycle, preimplantation testing), further highlighted the requirements for an accurate and efficient diagnosis, offering the greatest chance for an unaffected pregnancy with the lowest risk of misdiagnosis.

4.1.2.1 DM1 PGD protocol development

Since the expanded DM1 allele is refractory to PCR amplification, the DM1 mutation cannot be directly visualised, making diagnosis more difficult compared to diagnosis for other single gene disorders. Diagnosis of an unaffected embryo is indicated by detection of the affected individual's non-expanded allele, while absence of this non-expanded allele implies the presence of an unamplifiable expanded allele. Further difficulties present in cases where the two parental alleles in an embryo are of equal size, where it is impossible to confirm that the affected partner's non-expanded allele has been inherited. In these cases, diagnosis can be facilitated by the incorporation of linked markers and the detection or absence of the phase alleles.

Previously described PGD protocols have involved the co-amplification of linked markers, but in cases of patient uninformativity for the linked markers, unlinked markers have also been used for contamination detection or protocols have involved the amplification of only the mutated region by TP-PCR.

The unlinked polymorphic markers D21S1414 and D21S11, described in several single-gene PGD protocols, were tested during initial tests, but were found to be associated with low amplification and very high allele dropout rates. These protocols were tested but have not been included here, as they were not optimised to a level considered acceptable for clinical application. Other previously used linked markers, for example, D19S207 and D19S219, were also tested during preliminary genetic analysis for several of the patients. These markers, however, showed low patient informativity, presented difficulties with multiplexing, as well as yielding difficult to interpret stutter patterns even on genomic DNA analysis. Possibly for this reason, DM1 PGD protocols from other centres, using these two markers, have involved hemi-nested PCR reactions, with an extended time needed for diagnosis (Dean et al., 2001;Fiorentino et al., 2006). These markers were, therefore, not optimised for use in PGD in our department.

Intragenic markers such as the Alu insertion/deletion polymorphism have also been described but not applied to PGD, as, although they may be useful in identifying the expanded allele, they are not suitable for contamination detection (Mahadevan et al., 1993b). Other intragenic polymorphisms have also been identified within introns and noncoding regions of the *DMPK* gene. Some of these alter restriction enzyme recognition sequences and PCR assays have been developed for their detection.

However, these polymorphisms are not of use for PGD either, as they are due to single base changes and cannot allow for contamination detection (Mahadevan et al., 1993a).

The amplification of a commonly used linked marker, either *APOC2* or D19S112, along with the *DMPK* region was generally preferred for diagnosis in our group of patients. Protocols 1, 2 and 4 (DM1/*APOC2*, DM1/D19S112 and TP-PCR), as indicated in table 3.4, are similar to previously described protocols, with some modifications in the method of cell lysis and concentration of reagents in the PCR reaction. The TP-PCR protocol was used for couples that were uninformative at *DMPK*. The 5CTG repeat allele was present in 10/23 (43.5%) affected and in 17/23 unaffected (73.9%) individuals, and was shared in 9/23 (39%) couples. This presented problems with diagnosis for these couples, as TP-PCR could not safely detect the 5 CTG repeat homozygous embryos.

For couples who were fully informative for the *DMPK* region, the linked marker allowed detection of contamination, while providing back up diagnostic information, in cases where the phase allele was known. For couples partially informative or completely uninformative at the *DMPK* locus, only half or none of the normal embryos, respectively, could be identified by *DMPK* analysis alone. In these cases, diagnosis was based solely on assessment of the phase alleles. In this study a cross-over event was detected between *DMPK* and *APOC2* in two cases. This is related to the greater distance between DM1 and *APOC2*, compared to that between D19S112 and DM1 (Kakourou et al., 2007). A similar event has been reported in another PGD case for Charcot-Marie-Tooth disease type 1a, where recombination was detected in an embryo that was still considered unaffected and transferred, leading to an affected pregnancy (Gutierrez-Mateo et al., 2008). The detection of the crossover underlined the potential danger of making a diagnosis, solely on the result of the linked marker, and highlighted the importance of not using the relatively distantly linked *APOC2* as the sole marker to identify the *DMPK* mutation in semi-informative couples, as has been previously described (Piyamongkol et al., 2001b). The co-amplification of another linked marker, on the opposite side of the *DMPK* gene would provide extra information on the cross-over event, and could be used as an extra measure to prevent misinterpretation of the results.

Further protocol development focused on overcoming the problems of misdiagnosis associated with marker recombination, as well as the TP-PCR scoring difficulties of the

5 CTG repeat homozygous samples, in order to improve DM1 diagnosis and reduce the chance of misdiagnosis.

4.1.2.2 New diagnostic protocols for PGD for DM1

The above led to the development of two new protocols, not previously reported in the diagnosis of DM1. Our strategy was to devise a protocol that could exclude the presence of the mutant allele but also confirm the presence of the non-expanded allele. The latter was particularly important especially for the 5 CTG repeat homozygous embryos. The DM1/*APOC2*/D19S112 protocol (protocol 3) was a triplex PCR, amplifying the two commonly used linked markers, *APOC2* and D19SS12, which are on either side of the *DMPK* gene, along with the mutated region. The mTP-PCR protocol (protocol 5) allowed *DMPK* allele amplification both by standard PCR and TP-PCR along with amplification of a linked marker. In this way mTP-PCR permitted detection of the expansion and estimation of the repeat number of the non-expanded alleles by TP-PCR, detection of the 5 CTG repeat allele and other non-expanded alleles by standard PCR, detection of the phase allele of the linked marker to support the diagnosis and/or detection of contamination.

The availability of these protocols facilitated diagnosis for all patients. For these reasons the remaining optimised protocols (1, 1a, 2 and 4) are not currently being used in PGD for DM1 in our department. Following initial testing of parental DNA, either the DM1 triplex or the mTP-PCR protocol is selected for diagnosis, depending on couple informativity.

This has had major clinical implications for PGD practice and modified the course of treatment for all patients with DM1 undergoing PGD, by reducing the time required for initial genomic DNA testing and eliminating the protocol optimisation step. In previous practice, blood sample analysis of the prospective parents during the pre-PGD workup, for the identification of informative polymorphic markers, included testing an average of seven polymorphic markers per couple (range 4-16) in search of an informative one. This was followed by setting up protocols for multiplexing the informative marker with the *DMPK* region on genomic DNA, testing of additional family members if available, and single cell optimisation of the new protocol. Currently, genomic DNA testing

simply involves performing the DM1 triplex and mTP-PCR protocols, and can, therefore, be completed within a few days.

The approach to IVF/PGD treatment has changed since the beginning of this study (figure 4.1). Until recently, because of the long time required for protocol optimisation, protocol workup was initiated immediately after the initial consultation had taken place. Applications for funding, reproductive assessment and application to the Human Fertilisation and Embryology Authority (HFEA) were performed only after or near completion of the workup at the single cell level. Consequently, on several occasions, though a diagnostic protocol had been optimised, the patients failed to come through for treatment due to a diminished ovarian reserve, funding problems, or due to change of mind. Currently, the HFEA application is put forward when the source of funding is known or after the initial consultation when the patients feel certain that they wish to proceed with the treatment.

As a general rule, although reproductive assessment is crucial, it is not performed until after treatment funding and HFEA license have been granted, due to the considerable cost of these fertility tests. Protocol development is initiated after completion of these initial fertility checks and may generally increase the waiting time for treatment by a further 6 months to maybe over a year for new single gene diagnoses.

As the protocols for PGD of DM1 are available for diagnostic application almost immediately, the couple interested in undergoing PGD can proceed through the remaining treatment steps without further delays, taking overall approximately 6 months from referral to treatment for DM1 cases, rather than over 1 year, which is the case for other single gene disorders.

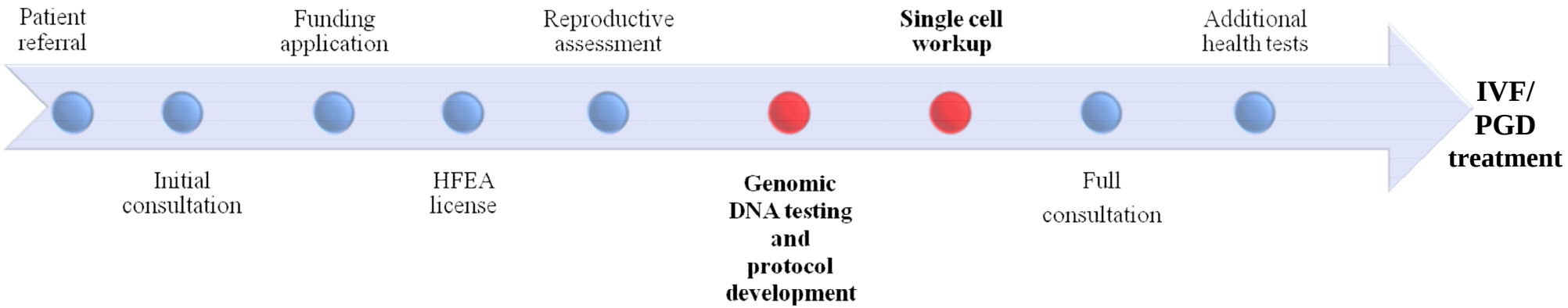

Figure 4. 1: Steps towards IVF/PGD treatment following patient referral. The funding and HFEA applications require a minimum of 5-8 months. Overall, excluding genomic DNA and single cell workup, the whole procedure can take 6-12 months from referral to the beginning of the IVF treatment (IVF cycle to egg collection: 6 weeks). This study reduced the PGD protocol workup time (genomic DNA and single cell work) for patients with DM1 to approximately 1 week, compared with new PGD single gene workups, which can take up to 6-12 months. As the HFEA license for PGD of DM1 has been obtained, HFEA application is also not performed for patients with DM1.

The mTP-PCR protocol could also be of use in PND. A recent study suggested that confirmation of diagnosis or prenatal testing for the detection of the DM1 expanded alleles should involve a two-step protocol, where standard PCR can be used for basic screening and, following that, the detected homozygous samples can be subjected to further analysis by TP-PCR to differentiate normal homozygotes from affected individuals. The suggested method was compared to other protocols available for detection of the expansion and was found to be the most rapid and least labour intensive, whilst also offering a straightforward interpretation of results (Falk et al., 2006). With the mTP-PCR protocol, the two-step protocol is performed in a single step, thereby saving further on time and cost. Finally, the incorporation of polymorphic markers can be extended to triplet-primed based protocols for the diagnosis of other conditions, such as FRDA and several types of SCA.

4.1.3 IVF/PGD practice at the UCL Centre for PGD

4.1.3.1 Embryo biopsy

4.1.3.1.1 One vs. two-cell biopsy

Several studies have attempted to define whether the removal of one or two cells during embryo biopsy has an impact on the efficiency of PCR-based or FISH-based diagnostic protocols, embryo development to the blastocyst stage, implantation or live birth rate.
Overall, testing two rather than one blastomere has been recommended as a safer procedure as far as the diagnostic efficacy goes (Van de Velde et al., 2000). One study investigating FISH diagnosis suggested that the overall diagnostic efficiency was similar between the one-cell and two-cell groups, although a false negative result was detected in the one-cell blastomere group (Emiliani et al., 2004). Goossens et al., (2008a) analyzed results from 592 ICSI cycles and concluded that the PCR-based diagnosis showed higher efficiency when two cells were biopsied, while no differences were detected for all cases with FISH diagnosis. Finally, in another recent study, the biopsy of a second blastomere was associated with decreased percentage of false positive results, as well as a reduced chance of misdiagnosis due to ADO (Dreesen et al., 2008).

Regarding embryo development, it has generally been shown that the more cells removed from human embryos, the fewer embryos develop to the blastocyst stage by day 5, although the removal of up to two cells has not been shown to have an adverse effect on later development (Hardy et al., 1990;Tarin et al., 1992). A similar observation has been made on mouse embryos, where it was indicated that when up to three cells were removed from 8-cell biopsied embryos, there was not a significant effect on the rate of blastocyst formation (Liu et al., 1993). On the other hand, Goossens et al., (2008a) suggested that removal of two blastomeres decreases the likelihood of blastocyst formation, but also observed that the embryo stage and grade on day 3 was a stronger predictor for embryo development compared to the number of cells removed. The same study concluded that live birth rates were not significantly different whether one or two cells were removed (data showed that for every 33 cycles there was one less delivery following removal of two blastomeres). This is also supported by Fiorentino et al., (2006), although data from other studies has generally been contradicting (Vandervors et al., 2000;Spanos et al., 2002;Pickering et al., 2003;Grace et al., 2006;Feyereisen et al., 2007). Finally, a study on the impact of aneuploidy screening indicated that the improved implantation rate and lower miscarriage rate, which is the aim of the screening technique, are not achieved when more than one cell is removed (Cohen et al., 2007).

The above arguments remain the topic of hot debate; there is, however, a tendency to support the one-cell biopsy policy, particularly because of the observed, though not significant, findings regarding live birth rate (Goossens et al., 2008a). Another important advantage of the one-cell biopsy is that it represents a lower work burden (Dreesen et al., 2008). It is, therefore, generally agreed that as long as a PGD protocol is available that can provide accurate diagnosis using a single blastomere, a one-cell biopsy should be recommended.

In this study, two cells were biopsied from all embryos with 6 or more cells on day 3. As mentioned previously, where either an informative linked marker was not included in the protocol, or when diagnosis involved use of the TP-PCR protocol, independent results from two cells from each embryo were required for diagnosis. In contrast, a diagnosis could be made on the results from one cell for couples that were informative for the mutation and one or two linked markers. Even in these cases, however, two cells were still biopsied. When one of the biopsied cells did not give a diagnostic result, a

diagnosis could be made from the results of the other cell. As a general rule, when either none of the biopsied cells gave a clear result or the protocol required a result from two cells, the embryos were rebiopsied and the protocol was repeated on the rebiopsied cells. Reanalysis of 10 out of the 11 DM1 cycles where the DM1 triplex (protocol 3) was used for diagnosis, was performed in order to assess the impact of the numbers of cells biopsied on diagnostic rate. The DM1 triplex was selected as it was the protocol most likely to allow a one-cell diagnosis, due to the incorporation of two linked polymorphic markers, and also because the protocols with TP-PCR analysis required results from two cells. The one cycle where a combination of two protocols was used for diagnosis, was excluded from the analysis. Results based solely on the first biopsied cell indicated that if one-cell biopsy had been performed, the diagnostic rate in these cases would drop from 73.4% to 53.1%, indicating a statistically significant difference ($p<0.05$, Fisher's exact test). This supports previous findings on increased diagnostic efficiency following two-cell biopsy.

Because of this impact on diagnosis, the one-cell biopsy has not been implemented in our department. The main priority has been to obtain a result from every single embryo, in order to increase the number of unaffected embryos available for transfer. For that reason, our practice has also included biopsy of additional cells where the first biopsy yielded anucleate or poor quality cells, as well as rebiopsy or delayed biopsy for slow-growing embryos. Therefore, in some cases (patient/cycle number 9/1, 8/2 and 23/1) embryos had up to four cells removed. In two of these cycles (9/1 and 23/1) there was no unaffected embryo for transfer following PGD. In cycle 2 of patient 8, where a combination of two protocols was used for diagnosis, there were two embryos with four cells biopsied each. Of these, one was a morula and was transferred following PGD along with another unaffected embryo, while the second embryo with four cells biopsied (a cavitating morula) was frozen and transferred in a subsequent cycle. No pregnancy was established in either case.

It is, however, expected that the new diagnostic protocols, due to their increased efficiency and improved diagnostic rate, will not only help reduce the number of embryos that need to be rebiopsied but also enable one-cell diagnosis for many more patients than was previously possible. The decision on the number of cells required to achieve a safe diagnosis may remain patient-specific.

Finally, it is worth mentioning that our approach in trying to achieve a diagnosis from every single embryo was independent of the number of the embryos available for biopsy from that specific cycle. This is contrary to practice from other centres, where when few embryos are available, embryo biopsy may be cancelled (Grace et al., 2006).

4.1.3.1.2 Number of pronuclei

Biopsy of five 0PN embryos, one 1PN and one 3PN embryo that had grown to the six or more cell-stage on day 3 was performed in cases where a limited number of embryos were available for testing (i.e. 2-4 embryos). These non-2PN embryos would be considered for transfer, if unaffected, only if no other embryos were available.

Despite the failure to detect pronuclei at the time of fertilisation check, which might be because of the embryo's accelerated or slow development, 0PN embryos can still cleave and develop to the blastocyst stage. Evidence from a FISH study has shown that 57% of 0PNs form diploid embryos, and more recently, 0PNs that cleave early have been associated with high chances of blastocyst development, implantation and pregnancy (Manor et al., 1996). These 0PN embryos can, therefore, be considered for transfer, but since it is unknown how many pronuclei were present, it is important to base the decision for transfer not only on the diagnostic result but also on other indicators such as the presence or absence of polar bodies, oocyte and embryo morphology, or other observations. For example, other events and observations detected in this study include fertilisation of a giant oocyte (cycle 3 of patient number 8), which is associated with numerical chromosomal abnormalities, or the presence of multinucleated blastomeres, which have been associated with poor development and lower clinical pregnancy rate (Tesarik et al., 1987;Jackson et al., 1998;Balakier et al., 2002). All four embryos where a multinucleate blastomere was biopsied were of poor quality on day 4: two embryos arrested, and the remaining two were at the 7 and 8-cell stage. One of these embryos was transferred but a pregnancy was not established. A similar association was observed for the embryos with binucleate cells, three of which gave a result and were all found to be affected. Lastly, micronuclei are known to be present in approximately 20-25% of embryos between days 3 -5 and are thought to possibly arise from chromosome loss although no significant observations were made on the four embryos in which micronuclei were observed in this study (Norppa and Falck, 2003;Chatzimeletiou et al., 2005).

Over 50% of 1PN embryos from IVF have been shown to be diploid, although the incidence of diploid 1PNs from ICSI cases is significantly lower (Staessen and Van Steirteghem, 1997). It has been suggested that ICSI 1PN embryos should not be transferred because of their higher chance to be parthenogenetically activated, an event which is, in turn, associated with chromosomal abnormalities and a risk of implantation failure (Sultan et al., 1995). This should not be a problem particularly with PCR analysis, as the presence of both parental genomes can be readily identified. It is, therefore, generally accepted that 1PNs may be transferred when no other embryo is available, as successful pregnancies with 1PN embryos have been reported (Feenan and Herbert, 2006).

In contrast to the above, the majority of 3PN embryos are predicted to have a triploid, greater than triploid, or mosaic chromosome content, which is associated with spontaneous abortion and neonatal death; transfer of triploid embryos should therefore be avoided (Feenan and Herbert, 2006).

The removal of the extra male pronucleus in a 3PN dispermic zygote and transfer of the resulting embryo leading to the birth of a healthy baby boy has been described (Kattera and Chen, 2003). This limited data is promising. Attempts to recover the abnormally fertilised oocytes may be particularly important to further increase the chances of an unaffected pregnancy, in cases of poor response to the IVF treatment and therefore, limited availability of oocytes and embryos, as has been described in patients with DM1. This practice should be combined with relevant preimplantation analysis (such as FISH) to confirm the absence of triploidy in the embryo.

In any case, assessment of fertilisation, embryo development and careful interpretation of data is vital for both 2PN and non-2PN embryos, as abnormalities are common even in apparently normally fertilised embryos (Delhanty et al., 1997). An example seen in our results is the detection of two paternal alleles in one embryo and the detection of only one parental genome in eight 2PN embryos, when the DM1 triplex and mTP-PCR protocols were used, which might indicate monosomy for chromosome 19.

4.1.3.2 Outcome of DM1 PGD cycles

Each couple completed on average 1.5 PGD cycles (fifteen couples had one cycle, four couples had two cycles and another four couples had three cycles each). For one couple, the results from their first cycle provided useful information regarding the phase, which had not been previously available.

The overall diagnosis rate of 74.12% (129/174) is relatively low compared to results from other studies, where chromosomal and single gene PGD diagnosis ranges from 80.7 to 94.3% (Pickering et al., 2003;Fiorentino et al., 2006;Feyereisen et al., 2007;Gutierrez-Mateo et al., 2008; Goossens et al., 2008b). Data specific on diagnosis for DM1 from two of the studies, indicates a diagnosis rate of 85% and 93.3% (Goossens et al., 2008b;Fiorentino et al., 2006).

It should be noted that our diagnosis rate quoted above includes diagnoses with older protocols no longer in use in our department. When analysis is limited to results with the two currently used protocols, 80% of embryos (52/65) were diagnosed with the DM1 triplex protocol and 91.6% embryos (22/24) were diagnosed with mTP-PCR, giving overall a diagnosis rate of 83% (74/89 embryos). It is expected that the total diagnosis rate will increase with future cases, since, following validation of the mTP-PCR protocol on several cycles, it should be the protocol preferred for diagnosis when possible.

This study also indicates the importance of carefully assessing the quality of each embryo and each biopsied blastomere. Diagnostic efficiency can be improved by ensuring that biopsied and amplified cells are morphologically sound with a clearly visible nucleus. Our data shows that only 4 out of the 33 blastomeres that were lysing during the biopsy but in which the nucleus was seen, failed to amplify. On the contrary, 41 out of 42 cells where a nucleus was not seen, failed to give a result following PCR. This is in keeping with previously reported findings (Piyamongkol et al., 2003;Gutierrez-Mateo et al., 2008).

Out of the 45 embryos that remained undiagnosed, twenty-seven gave inconclusive results, ten showed total amplification failure and eight embryos could not be diagnosed due to the biopsied cell result indicating the presence of a single parental genome. Out of the 27 embryos with inconclusive results, the lack of diagnosis was due to couple uninformativity in 51.9% of the embryos (14/27), which included cases completed prior to the mTP-PCR protocol becoming available, contamination in 5/27 (18.52%), “other” findings, such as detection of two paternal alleles in 4/27 (14.81%), allele dropout in

3/27 (11.11%), or, in one of the embryos, result from only one cell when using a protocol requiring results from two cells for diagnosis (3.7%).

The implantation rate (IR) was 28.2% (11/39 embryos transferred). This is comparable to results from other studies with IR ranging from 23-28% (Pickering et al., 2003;Fiorentino et al., 2006;Grace et al., 2006;Goossens et al., 2008b; Gutierrez-Mateo et al., 2008).

Considering that two cells were biopsied from the majority of embryos, and over two cells were biopsied from several embryos, it is noteworthy that our IR is much higher than the reported rate of 12.8% and 14%, from two other groups where two-cell biopsy was also performed (Feyereisen et al., 2007;Vandervors et al., 2000). Finally, this study indicates a pregnancy rate per embryo transfer of 36.5%, including the two transfers of frozen-thawed embryos, where no pregnancy was established. This rate is high considering that pregnancy rates per ET from other studies have ranged between 21.5-43.4%, with most being ≤ 33% (Grace et al., 2006;Fiorentino et al., 2006;Feyereisen et al., 2007;Dreesen et al., 2008;Goossens et al., 2008b; Gutierrez-Mateo et al., 2008).

Pregnancies were established even from embryos considered to be of poor quality. Four of the eleven embryos that implanted had eight or fewer cells at the time of transfer, and three of the implanted embryos, including a 5-cell embryo, had been rebiopsied. Post-natal DNA analysis in case no.10 revealed that it was the slow growing rebiopsied embryo, that had had two cells removed, that implanted. This differentiation could not be made for case no.1 as both of the embryos transferred, one of which was rebiopsied and the other not, had given the same genotype on analysis.

4.1.3.3 Follow-up analysis

4.1.3.3.1 Analysis of spare embryos

Despite relevant counseling, none of the couples opted for prenatal diagnosis, so as not to risk losing the precious pregnancy achieved following the physically and emotionally stressful IVF/PGD cycle. This further underlines the importance of ensuring diagnostic accuracy during PGD.

Confirmation of diagnosis was routinely performed for all embryos that were affected or unaffected but unsuitable for cryopreservation, in order to test the validity of the assessment and estimate the chance of misdiagnosis.

Overall, 86 embryos were available for confirmatory analysis, of which 53 had been diagnosed as affected or unaffected during PGD, 20 had given an inconclusive result, 6 had no result and 7 had not been biopsied. In most, but not all, cases, confirmation involved application of the same protocol that was used for genotyping and blastomere diagnosis, although some follow-up analyses included a combination of the DM1 triplex and mTP-PCR protocols on single cells or blastomere clumps. When possible, depending mainly on the number of cells present, both a single blastomere and a blastomere clump were obtained for reanalysis from each embryo. In cases where the embryo quality was poor or when disaggregation was difficult because of the embryo being very compact, the embryo was tubed whole.

Forty-seven out of the 53 embryos where a diagnosis had been obtained were confirmed during follow-up (7/8 unaffected and 40/45 affected). From the 6 embryos where the diagnostic result was not confirmed, one unaffected embryo and three affected embryos gave an inconclusive result during reanalysis. The reasons for these embryos being inconclusive were the presence of a cell showing amplification from one parental genome only (one unaffected embryo), ADO (one affected embryo) and contamination (two affected embryos). The two remaining affected embryos, a three-cell and a four-cell embryo, failed to amplify on reanalysis. Overall, the reanalysis result concurred with the primary diagnostic result, i.e. no false negatives or false positives were detected.

The embryos that had not given a result during PGD and the embryos that had not grown to a stage to allow biopsy on day 3 were tubed whole during reanalysis, and all of them were successfully analysed and yielded an affected/unaffected result. This provided additional information on the number of affected and unaffected embryos obtained from this group of patients. From the twenty embryos tested that had been

inconclusive on diagnosis, ten remained inconclusive during re-analysis, one did not give a result (9-cell embryo), while a diagnosis was achieved for the remaining nine. Most of the embryos that remained inconclusive despite reanalysis came from couples sharing marker alleles (7 embryos), while two other embryos showed evidence of contamination in the biopsied cells and one embryo had ADO. Use of the mTP-PCR protocol in cases of couple uninformativity would reduce the number of undiagnosed embryos due to inconclusive results.

The data from follow-up analysis using MDA, though limited, gave very poor results. Further investigation and potential protocol optimisation was not deemed necessary in this study as the available optimised protocols did not only provide faster results but also combined direct mutation detection with linkage analysis.

4.2 Investigation of DMPK repeat transmission

4.2.1 Allele transmission

Preferential transmission of large non-expanded alleles at the *DMPK* locus has been reported in offspring and human preimplantation embryos, however, results regarding transmission of the expanded allele have been contradicting (introduction section 1.2.3.1). Investigation of transmission of the *DMPK* repeat from the affected parent to the preimplantation embryo has not been previously available. Allele transmission was investigated by analyzing data in three ways. First of all, observing the number of affected *vs*. unaffected embryos on diagnosis. Secondly, separating parental alleles into ‘small’ or ‘large’ (expanded or the larger non-expanded) and finally, by grouping of parental alleles into different repeat classes to detect potential differences in transmission depending on allele size.

In summary, transmission ratio distortion (TRD) was not observed from the above analysis; our results do not support preferential transmission of the expanded repeat allele to the preimplantation embryo, agreeing with the results from the two prenatal studies (Zunz et al., 2004;Martorell et al., 2007). This is also indicated by the non-significant difference in the number of embryos diagnosed as affected and unaffected. It

should be noted, however, that the level of expansion was unknown in most cases and this might also influence transmission. Previous investigators similarly reported no significant TRD for non-expanded 5-18 repeat alleles, but detected preferential transmission of 19-37 repeat alleles (Dean et al., 2006a). In this study there was information from a total of 30 transmissions involving alleles of 19-37 repeats, and in these individuals, the second repeat allele was expanded. Statistical analysis did not indicate a significant difference in the transmission of these repeat classes (binomial test) , however, more data would be necessary due to the small sample size.

A similar analysis was performed focusing on results from patients with a previous history of an affected pregnancy, but, again, there was no statistically significant difference between the number of embryos diagnosed affected or unaffected from this group.

4.2.2 Instability of repeat transmission

In a previous study, intergenerational instability at the level of human gametes and preimplantation embryos was detected by use of a specific PCR for long fragments, followed by Southern blot analysis for detection of the expansion, as well as a second round PCR for detection of the smaller alleles. In this way significant increases in repeat number were detected in oocytes and embryos from female patients with DM1, while smaller increases were detected in spermatozoa and embryos from male patients (De Temmerman et al., 2004). It is noteworthy, however, that the technique used had limitations in detecting large expansions. A subsequent study reported the use of TP-PCR for sizing of the non-expanded as well as the expanded *DMPK* repeat alleles, in both oocytes and embryos, based on the specific ladder patterns produced following amplification from each sample. In particular, expansions were sized as “maternal-type”, when the results were similar to amplification from maternal lymphocytes, “intermediate” and “congenital”, corresponding to precisely 170-190bp, 200-240bp, and >250bp on F-PCR analysis, based on results from two patients (Dean et al., 2006b). A variable degree of expansion was detected in preimplantation embryos, with several indicating expansion in the congenital range. Additionally, instability was seen during transmission of non-expanded 19-37 repeats from the father to the preimplantation

embryo, in keeping with previous observations of instability in transmission of normal alleles at the level of offspring (Dean et al., 2006b).

In this study we attempted to optimise the TP-PCR technique in order to reproduce similar experiments that would enable detection of instability in our larger group of patients with DM1 undergoing PGD. The results presented here, however, are contradicting to the previous study. From our experience, TP-PCR was unable to size expansions even when comparing control genomic DNA samples with small, medium, larger and congenital range expansions. This observation was supported by a recent study concluding that TP-PCR could only accurately size alleles of up to 50 repeats (Falk et al., 2006). We, therefore, conclude that Southern blotting still remains the best way to estimate the triplet repeat size.

We also noted that, in affected samples, TP-PCR amplification was extremely variable even amongst single lymphocytes of the same individual, while the previous study reported this to be consistent. Differences were also detected when blastomeres from the same affected embryo were amplified, which could, however, also be due to differences in cell quality. Regardless of this variation, we described TP-PCR amplification products in several day 3 embryos as being larger than any of the patient lymphocyte results. Several day 5 embryos showed bigger amplification products compared to their day 3 result, which would suggest a case of mitotic postzygotic expansion. Pre- and post-zygotic expansion of the CTG repeat has also been proposed for Fragile X, however, the significance of the above observations remains unverified (Ashley and Sherman, 1995).

On the other hand, TP-PCR was more accurate in sizing non-expanded repeat alleles. This allowed to detect a potential allele instability in 3/37 (8%) unaffected blastomeres analyzed. Similar changes in the number of repeat units during transmission (>7%) have been reported (Dean et al., 2006b).

4.2.3 Embryo development and CTG repeat allele transmission

Embryos were generally scored by the embryologists on day 2, day 3, pre- and post-biopsy, day 4 and/or day 5. Following diagnosis and follow-up, development of all known unaffected embryos was compared to the development of affected embryos. To allow a fair comparison, only embryos biopsied on the morning of day 3 were included

and embryos that had been rebiopsied were excluded from the analysis. In addition, most embryos had one to three cells removed, but the majority of them were in the two-cell category. Development of unaffected embryos with two cells biopsied on day 3 was compared to development of the affected embryos that had two cells biopsied on day 3. Results indicate that a greater number of affected embryos developed to the morula or cavitating morula stage by day 4, compared to the number of unaffected embryos at the same stage.

This observation is significant as an increased survival of affected DM1 embryos might indicate a mechanism by which expanded alleles are maintained in the population. Another study has shown that the presence of large non-expanded alleles, of 19-38 repeats, does not influence preimplantation development however it could be that different sized repeats might have a different impact on the embryo (Dean et al., 2006a). It has also been reported that expanded cells of lymphoblastoid cell lines present a growth advantage and faster cell proliferation over cells with smaller expansions (Khajavi et al., 2001). The accumulation of more data from DM1 PGD cases is necessary to further support these findings at the preimplantation stage.

In conclusion, the first part of this study (aims 1 and 2) enabled the development and clinical implementation of new universal protocols that can be routinely applied for the diagnosis of DM1 at the preimplantation stage. The results from our 28 DM1 IVF/PGD cycles, the largest number of cycles in the UK, demonstrate that PGD for DM1 is a practical, reliable and effective option for couples to avoid passing the disorder on to their children without the need for termination of affected pregnancies (Kakourou et al., 2008). The implantation and pregnancy rates have been very encouraging and this is fundamental to the PGD program. PGD for DM1 can be applied within one month after patient referral and should be suggested as another alternative to PND during counselling of couples with increased genetic risk. Furthermore, this study has provided additional information regarding the impact of the number of cells biopsied, which has recently accumulated a lot of debate. Since completion of analysis in this study, several of the patients have had additional IVF/PGD cycles. In particular, patients 8 and 23 have now had a total of 4 cycles each and patient 19 has had a total of 3 cycles. This emphasizes that these couples are willing to go to great lengths to avoid passing on DM1 to their children and underlines the importance of obtaining an accurate diagnosis for every single embryo to improve the chances of a successful IVF/PGD cycle. Both of

the new optimised protocols, the DM1 triplex and the mTP-PCR, involve a single PCR amplification step. Therefore, these methods enable a faster and significantly cheaper diagnosis compared to other described attempts for the establishment of universal PGD protocols, that involve an MDA approach (Burlet et al., 2006;Renwick et al., 2006).
This study has also provided information from 28 couples regarding repeat transmission from affected parent to embryos at the preimplantation stage, mostly affected female to embryo and unaffected male to embryo. Transmission ratio distortion was not observed, though a growth advantage of DM1 affected vs. unaffected embryos was detected. Implementation of the new protocols in clinical practice will allow accumulation of data to further support the findings described in this study regarding allele transmission and embryo development.

4.3 Expression work

4.3.1 Sample processing for microarrays

The difficulties associated with obtaining and generally working with human oocytes and embryos pose limitations to research in this field. Several studies have been conducted using poor quality oocytes and embryos that would be discarded during IVF, or oocytes that were injected with sperm but failed to fertilise, similarly of no use in IVF. The human oocytes used in this study were immature at the time of collection, and, therefore, unsuitable for IVF treatment, but matured in culture prior to processing. The human embryo blastocysts had been cryopreserved and were donated for research following the couples' decision not to use them for embryo transfer.

Microarray work with such precious samples requires great attention to ensure that an accurate expression profile is obtained. Careful evaluation of the developmental stage of the sample is essential prior to sample selection, pooling and processing, as well as complete removal of the zona pellucida, to prevent nucleic acid contamination. Overall handling should be swift to prevent RNA degradation. Oocyte and embryo samples were stored at -80°C until enough were collected to be able to pool them in groups of three. Though a microarray result can also been obtained from a single oocyte, many

transcripts were not detected due to the lack of sensitivity for low template mRNA. For that reason, it has been recommended that a minimum of three oocytes are pooled together for microarray experiments (Jones et al., 2007).

For experimental designs that involve comparison of two groups for differential expression testing, such as comparison of the blastocyst to the human MII oocyte in this study, it is indicated that a minimum of five biological cases per group should be analyzed (Pavlidis et al., 2003). The more biological samples tested, the greater the significance of the microarray results. Pooling of mRNA from biological replicates increases the biological sample size without the need to utilise more arrays. The samples pooled together were from different donors in order to overcome individual variation. In this way inter-individual variation cannot be assessed, but this practice generates an overall representative gene expression profile from the tested sample. Biological replicates are considered essential and should be preferred to technical replicates (testing of mRNA from a single sample on multiple arrays), as they allow to not only estimate the effects of measurement variability but also account for any biological differences (Allison et al., 2006).

Additionally, samples were processed a few at a time in order to minimize the overall handling time during the RNA isolation particularly in view of the long protocol for the RNA amplification procedure. Careful planning of each experimental step was essential in order to avoid unnecessary delays and minimize freezing-thawing of the samples. The triplicate array experiments for blastocyst-oocyte comparison were all performed using arrays from a single batch. All arrays were processed at the same time to avoid influences on the results by other unknown factors.

The experience with single cell work for PGD and the availability of the PGD laboratory set-up, designed so as to reduce the chances of DNA contamination, along with the incorporation of practice guidelines specifically for RNA work, provided the necessary environment for successful processing of the samples to be used for microarrays. Optimal conditions for working with RNA were first tested on lymphocyte samples prior to commencing the microarray work. Good practice was then confirmed by the Bioanalyser results for the samples used for microarrays, indicating good RNA integrity and the absence of DNA contamination.

Reported estimates of the total RNA amount per human oocyte range between 55-330pg (Neilson et al., 2000;Dobson et al., 2004;Kocabas et al., 2006). In our study RNA concentrations extracted from pooled samples ranged between 48-78pg/μl, on average (from all samples) 58.6pg/μl (14μl of eluted volume for each sample set of three

oocytes), i.e. 273pg/oocyte (0.27ng/oocyte). The amount of RNA from the human blastocyst sets (the DM1 affected sample excluded) ranged from 63-393pg/µl, with an average from all readings, of 223.3pg/µl, i.e. 1042pg/ blastocyst (or 1ng/blastocyst).

As microarrays require at least 10µg RNA for hybridization, the samples were amplified using a two-round linear RNA amplification protocol, which amplifies more than 100,000 times the initial RNA input, achieving a new RNA concentration range for pooled oocyte samples between 22.9ng/µl to 296ng/µl and 59.5-232.8 ng/µl for the embryo samples.

It is noteworthy that the lowest concentrations were obtained from oocytes and embryos processed in the early part of our experience, indicating the importance of careful set up and expertise with each new experimental procedure. The three sets of oocytes and embryos for which hybridization of 10µg was possible for the microarrays were used as biological replicates. The amplification protocol used has been previously validated by other investigators and the amplified material is thought to be representative of the original samples (Bermudez et al., 2004;Patrizio et al., 2007).

This study overcame limitations, such as the use of discarded human oocytes that failed to fertilise, as well as the lack of sufficient biological and technical replicates. In addition, the use of a very sensitive microarray platform in this study, allowing the interrogation of over 29,000 genes has overcome the limited coverage of several previous array experiments (Kocabas et al., 2006).

4.3.2 Microarray results analysis: general expression profiling

The differences in the number of probes detected between the three replicates for oocytes and blastocysts can be attributed to the random pooling of samples from different donors, with a different genetic background, to differences in the microenvironment of each oocyte and embryo, the time required for collection and tubing for each sample as well as differences in RNA quality and amplification.

Overall, 11679 probes and 13118 probes were detected in all three replicates of oocytes and blastocysts respectively. As only the probes common between all samples were considered for further analysis we assume that sample-specific differences are eliminated.

Failure to detect expression from cumulus-cell specific genes in the oocytes and blastocysts was taken as a confirmation that all samples were successfully denuded of

the zona pellucida. Examples of these included *PTGS2*, *PTX3*, *GREM1* and *STAR*, which are downstream targets of the oocyte-secreted factor GDF9 and have been described as cumulus cell markers of oocyte competence. Other cumulus cell markers that were undetected included the KIT ligand (KITLG), which is produced by the granulosa cells and binds to its receptor on the oocyte surface to stimulate oocyte growth, as well as epidermal growth factor (EGF)- like proteins of the granulosa cells, amphiregulin and epiregulin, that have been associated with oocyte maturation (Mehlmann, 2005;Cillo et al., 2007;Li et al., 2008). The levels of expression of several genes previously investigated were also assessed in our sample data.

The majority of genes detected in each sample broadly belonged to the nucleic acid and protein metabolism categories and most genes with highest expression in both samples, excluding the unclassified function genes, were involved in protein metabolism. Additionally, the oocyte also showed generally high expression in the categories of nucleic acid metabolism and cell cycle. Despite the main differences, however, several genes showed similarly very high levels of expression in both samples, such as the heterogeneous nuclear ribonucleoprotein A1 (HNRPA1) involved in mRNA splicing, and the ribosomal protein L7a (RPL7A), involved in protein biosynthesis, suggesting a fundamental role of these transcripts in preimplantation development.

When the gene expression was compared between the two samples, taking the human oocyte as the control, 4910 genes showed statistically significant differences in expression ($p<0.05$). Of these, 2652 genes were over expressed in the blastocyst and 2591 genes were under expressed in the blastocyst compared to the oocyte. Excluding the genes of unclassified function, the majority of the remaining genes showing higher expression in the blastocyst belonged to the protein biosynthesis category, while other important categories included intracellular protein traffic, transport, and immunity and defense. On the other hand, genes that showed higher expression in the oocyte are associated with nucleic acid metabolism, signal transduction, developmental processes or the cell cycle.

The protein metabolism (higher expression in blastocyst) and nucleic acid metabolism (higher expression in oocyte) categories were investigated in more detail. Genes over expressed in the blastocyst were involved in protein biosynthesis, protein folding, translational regulation, protein complex assembly or amino acid regulation, while most

protein modification genes showed higher expression in the oocyte. From the nucleic acid binding category the blastocyst showed higher expression for genes that had to do with pre-mRNA processing, rRNA and tRNA metabolism, purine and pyrimidine metabolism, while the oocyte showed higher expression for genes involved in DNA processing, chromatin packaging and remodeling, RNA localization or metabolism of cyclic nucleotides.

The genes that showed over a 10-fold difference in gene expression between the two samples were further analyzed by molecular function. It was found that the blastocyst showed over expression of ribosomal proteins and translation factors, while the oocyte showed over expression of chromatin-binding factors, transcription factor, and DNA methyltransferases.

Overall, the oocyte was rich in genes involved in DNA metabolism, while the blastocyst was mostly active in protein metabolism. The initial analysis supports previous findings that human oocytes, though transcriptionally silent, are well equipped with transcripts and proteins in order to be able to support chromatin remodeling during fertilisation as well as early preimplantation development until initiation of transcription in the embryo (Kocabas et al., 2006). On the other hand, the human blastocyst is very active in synthesizing proteins, but also shows high activity in genes that have to do with DNA or chromosomes. This has also been previously suggested to be associated with the higher number of nuclei in the blastocyst sample (Wells et al., 2005b).

4.3.3 Oocyte and blastocyst-specific genes

Once the genes expressed in a tested sample are known, the next important goal is to understand the impact of their expression and what function they contribute to preimplantation development.
For that purpose, we proceeded to investigate the genes that are uniquely expressed in oocytes and blastocysts, as this might reveal which genes play a critical role in maturation, fertilisation and development and which genes are expressed as a result of embryonic genome activation. Previous comparisons of oocytes, of different maturation stages, with their surrounding cumulus cells, and investigation of genes expressed in embryos at different developmental stages have provided some additional intriguing information (introduction section 1.3.2). The investigation and comparison of oocyte and blastocyst-specific genes, however, possibly indicating genes with important roles pre- and post- embryonic genome activation, has not been previously reported.

A total of 1909 genes were uniquely expressed in the oocytes, while 3122 were uniquely expressed in the blastocyst samples. Oocyte-specific genes included the zona pellucida glycoproteins (ZP1, ZP2, ZP3, ZP4) and members of the TGF-beta superfamily, such as *GDF9* and *BMP15*, that are known to play a role in follicle growth, maturation and cumulus expansion (McKenzie et al., 2004). In addition, known germ-cell specific genes were included in this category, such as factor in the germline alpha (*FIGLA*), deleted in azoospermia-like (*DAZL*), v-mos Moloney murine sarcoma viral oncogene homolog (*MOS*), nucleophosmin/nucleoplasmin 2 (*NPM2*) and H1 histone family, member O (*H1FOO*). Genes unique in human blastocysts included annexins A2 and A3 (*ANXA2*, *ANXA3*), gap junction protein, alpha 1 (*GJPA1*), GTP binding protein 4 (*GTPBP4*), ATPase H+ transporting, lysosomal accessory protein 1 (*ATP6AP1*).
Uniquely expressed transcription factors represented an important molecular function category in both sample types.

When grouped by level of expression, 349 of the oocyte-specific genes (18.3%) and 431 of the blastocyst-specific genes (13.8%) showed high expression levels.
Amongst the oocyte-specific genes, high expression was detected for genes regulating glycogen metabolism, lipid metabolism and calcium homeostasis, while important molecular function categories involved mitochondrial carrier proteins, cyclases and the microtubule family of cytoskeletal proteins. On the other hand, the blastocyst-specific

genes were associated with oxidative phosphorylation, glycolysis, sterol metabolism, and were rich in RNA-binding proteins, methyltransferases, gap junction proteins and intermediate filaments.

Experiments on porcine and bovine oocytes have demonstrated the role of lipid metabolism in the oocyte and the use of triglycerides (TG) as endogenous substrates for the generation of ATP (Sturmey et al., 2006). TG is stored as lipid droplets, which have been shown to be in close proximity to the mitochondria, where the free fatty acids are transported in order to be oxidized by beta-oxidation and the TCA cycle. Mitochondria actively relocate and surround the newly formed pronuclei, following fertilisation of the human oocyte, to concentrate ATP and calcium to support normal developmental processes (Sousa et al., 1997;Sun et al., 2001). In mouse oocytes, the movement of mitochondria is mediated by microtubules, which also justifies the high expression level of the microtubule family of cytoskeletal proteins in these cells. The pre-existing oocyte mitochondrial proteins and transcripts are necessary to generate ATP until new biosynthetic activity develops following embryonic genome activation. Their significant role is also underlined by the fact that mitochondrial DNA defects, either pre-existing or age-related, have been associated with reduced meiotic competence and fertilizability of the oocyte as well as developmental failure in the preimplantation embryo (Van et al., 2000). Our findings support that the human oocyte has all the machinery required to support the synthesis of cAMP, which controls nuclear maturation (Kawamura et al., 2004;Richard, 2007). This includes receptors, guanosine 5’-triphosphate-binding (G) proteins, cyclases (which synthesize cyclic nucleotides) and phosphodiesterases (degrading cyclic nucleotides), which were found to be amongst the oocyte-unique genes and also of high expression level. The identification of the above unique oocyte genes is significant and may be further investigated to provide information on the oocyte quality (Van et al., 2000).

Highly expressed human blastocyst genes were associated with oxidative phosphorylation as well as glycolysis. These processes control ATP generation during pre-compaction and cavitation stages respectively (Van et al., 2000). Therefore, these genes probably exist from the point of embryonic genome activation and onwards. The blastocyst, contrary to the oocyte, showed high expression in genes involved in the conversion of acetate to cholesterol. The sterol synthetic pathway has also been shown in mouse oocytes and embryos not to be operative until the blastocyst stage of development. These processes were not significantly detected in the oocyte samples, which is an expected finding as the oocytes can obtain the necessary nutrients from the

surrounding cumulus cells. For example, BMP15 has been shown to stimulate the glycolytic activity in granulosa cells since the oocytes are not able to generate ATP (Sugiura et al., 2007). In addition, mouse oocytes can stimulate cholesterol synthesis in cumulus cells, which in turn supply the oocytes with cholesterol (Su et al., 2008).

Finally, highly expressed blastocyst genes were involved in the platelet derived growth factor (PDGF) pathway, which plays a critical role in cellular proliferation and metabolism, the Rho GTPase control pathway that regulates cytoskeletal changes occurring during cell growth and development and the Integrin signalling pathway, also important in actin reorganization. Most oocyte genes, on the other hand belonged to the Wnt signalling pathway, important in recruitment of membrane proteins and general transcription regulation.

4.3.4 Investigation of housekeeping gene expression

Housekeeping genes are highly and stably expressed in a variety of tissues and cell types. Because of their role in regulating basic cell processes, they generally provide some information on the quality and function of a cell, while their expression might be indicative of the expression of a number of other genes. Additionally, housekeeping genes can be used as endogenous standards for normalization of gene expression data across various samples. This approach requires careful validation of the housekeeping gene expression under different developmental stages and experimental conditions, involving, to ensure accuracy, a rather large number of test samples as well as biological and technical replicates.

The above are difficult with regards to human preimplantation embryo work, where there is limited availability of material. For that reason, several studies have attempted different methods of normalization, however, none of them is ideal for working with minute amounts of RNA. The absolute RNA quantification is impractical for sizing small samples, while the approach of adding exogenous template has been challenged for competing for enzyme and nucleotides with the endogenous sequence, while at the same time increasing the cost and introducing extra procedures (Huggett et al., 2005;Jeong et al., 2005;Mamo et al., 2007;Mamo et al., 2008).

In this study, microarray data obtained from human oocytes and embryos was analyzed for the identification of the level of expression of genes with a potential housekeeping

role. The analysis has identified the genes that are highly expressed in these samples, which can be taken as an indicator of the different pathways regulating these complex developmental stages, and also identified several potentially stably expressed genes that may be further validated.

Five hundred and sixty (560) genes were analyzed in total. Most genes showed high expression levels in both samples, and 90.9% of the high expression genes of the oocyte remained of high expression in the blastocyst sample. The majority of these were ribosomal proteins.

Two hundred and eleven (211) of all detected genes showed significant differences in expression between the two samples. The majority of them, 155 genes, were up regulated in the blastocyst, while the remaining ones, 56, showed higher expression in the oocyte. Most gene groups included both up- and down-regulated genes from the oocyte to blastocyst stage. Differentially expressed genes from the tRNA synthetase, hnRNP and snRNP categories were all upregulated in the blastocyst, while differentially expressed genes from the nuclear pore complex category were all found to be down regulated in the blastocyst sample. This also underlines the importance of nuclear transport and cytoskeletal organization in the oocyte and provides another list of genes with potential significance for the normal function and development of the human oocyte. On the other hand, the snRNPs and hnRNPs, involved in mRNA splicing and protein methylation and modification along with tRNA synthetases controlling amino acid activation and general tRNA metabolism, play a significant role in the growing blastocyst.

Previous experiments have demonstrated major differences between the housekeeping genes validated from studies on adult tissue and those from embryonic stem cells or stages of preimplantation development. Overall, the most frequently used housekeeping genes, *GAPDH* and *ACTB*, have been determined from experiments involving adult tissues and cell lines. These have shown very high variability both on embryonic stem cell studies and during human preimplantation development. *ACTB* showed an over 10-fold increase in expression in the human blastocyst as compared to the human oocyte. This difference has also been identified in other human and mouse studies (Wells et al., 2005b;Mamo et al., 2007). In particular, *ACTB* has been shown to fluctuate between the 5-10 cell to the morula stage and then rapidly increase in the morula and the blastocyst (Wells et al., 2005b). In one recent study, comparing the expression of genes on day 3 human embryos to their expression in human oocytes, *GAPDH* was used as a reference gene to normalise expression data to control mRNA recovery and reverse transcription

efficiency; its expression was assumed to be constant, without prior validation (Dobson et al., 2004). In this study, however, *GAPDH* was found highly up regulated in the human blastocyst compared to the human oocyte (table 3.30). *ACTB* was also suggested as a control in a mouse preimplantation development study (Willems et al., 2006). From the current analysis, amongst other commonly used housekeeping genes, eukaryotic translation elongation factor 1 epsilon 1 (*EEF1E1*, protein biosynthesis) and ubiquitin C (*UBC*, proteolysis) showed the most stable expression, with *UBC* showing overall higher expression signals in both samples.

A recent study on immature oocytes and embryo developmental stages using cryopreserved human embryos, recommended the use of the eukaryotic translation elongation factor A (*EEF1A1*) and the proteasome subunit beta, type 6 (*PSMB6*), associated with translational regulation and proteolysis respectively, as reference genes in human preimplantation studies (Zhang et al., 2008). Other preimplantation studies on mouse, rabbit and bovine embryos have indicated the comparative stable expression of *PPIA*, *CHUK*, *TBP*, Histone H2A, *H2AFZ*, *YWHAZ* and *HPRT1* (Robert et al., 2002;Falco et al., 2006;Willems et al., 2006;Mamo et al., 2007;Mamo et al., 2008).

From all of the above genes, in our analysis *CHUK* gave a stable signal between the oocytes and blastocysts, but the remaining genes do not seem to be suitable as control genes for human preimplantation studies as they showed significant expression variability.

4.3.5 Assessment of gene expression for other functional pathways

DM1 is a multifactorial disease and affects many different pathways, including some that play a significant role during preimplantation development, such as methylation. Other genes, for example genes encoding components of the microRNA processing machinery, also known to play a critical role in oocyte maturation and embryo development, have not been previously investigated at the human preimplantation stage (Murchison et al., 2007;Tang et al., 2007).

MicroRNAs are short, non-coding endogenous RNAs that bind to conserved sequences within the 3' untranslated regions of specific mRNAs and inhibit their translation, regulating, in this way, the expression of up to one third of human genes (Lewis et al., 2005). MicroRNAs are derived from long primary transcripts transcribed by RNA polymerase II (pri-miRNA), cropped by nuclear RNAse III Drosha into 70 nucleotide-long pre-miRNAs, exported out of the nucleus by *XPO5* and cleaved by cytoplasmic RNAse III DICER into the 17-25 nucleotide-long miRNA duplexes. One strand of the miRNA duplex is incorporated, along with the Argonaute protein Ago2, into the RNA-induced silencing complex (RISC), which scans cellular mRNA in an attempt to locate the miRNA target (Mtango et al., 2008;Schmittgen, 2008). A recent study on human embryonic stem cells (hESCs) has revealed that most of the hESC miRNAs are located on chromosomes X and 19 (Cao et al., 2008).

In this study, the expression of genes from this pathway was investigated in the human MII oocytes and embryos. Significant observations included strong maternal expression of *DICER1* and significant downregulation of *GEMIN5*, *SIP1* and *TNRC6B* at the blastocyst compared to the oocyte stage. The expression of *RNASEN* was high in both oocytes and blastocysts. On the other hand, *YBX1*, *GEMIN4*, *MOV10* and the exportin genes (*XPO1*, *XPO4*, *XPO5*) were all significantly up regulated in the blastocyst. Several genes were only detected at one of the two stages.

The above results from the human samples can be compared with studies on mouse and rhesus monkey preimplantation oocyte and blastocyst embryo development regarding the level of expression of these genes (Zheng et al., 2004;Mtango et al., 2008).

Some similarities and differences are observed in the expression of these genes between the three species. The expression patterns of *RNASEN*, *DICER1*, *GEMIN5* and *TNRC6B* are similar to the results from the mouse study, while upregulation of *YBX1* at the blastocyst stage was also detected in the rhesus monkey study. *XPO4* increased during development in all three species. *PIWIL1* was not detected in either oocytes or blastocysts, similarly to the other studies. *MOV10*, which was not detected in the monkey, was found highly up regulated in the human blastocyst, suggesting that some of these processes might be controlled differently in different species (Zheng et al., 2004; Cui et al., 2007;Mtango et al., 2008).

Data analysis also identified the expression level of methyltransferases and other DM1-associated genes, thereby providing a useful foundation of information for experiments on DM1 affected samples, in comparison to unaffected samples.

Expression of the *DMPK* gene was not detected in the oocyte or blastocyst samples tested in this study. However, a previous study reported detection of maternal *DMPK* transcripts in two unfertilised oocytes and detection of transcripts from the paternally inherited allele in 16 out of 19 preimplantation embryos, of up to four blastomeres each, donated by IVF patients (Daniels et al., 1995). It is unknown whether expression of *DMPK* may serve a specific function at this early developmental stage, however, considering the potential differences in development between affected and unaffected embryos suggested in this study, this would be something interesting to further investigate. Additionally, the above give further emphasis to the requirement for continuation of the microarray experiments using more sensitive analyses, to investigate the absence of several transcripts. Lack of amplification or underrepresentation of some transcripts may be associated with the oligo(dT) amplification step performed for generating the microarray data (Bell et al., 2008).

In summary, this work confirmed several previously described findings from studies on preimplantation development of human and other species and provided the opportunity to study the level of expression of a large number of genes, not previously investigated, in healthy human oocytes and embryos. The identification of genes that maintain high expression levels during the initial and latest stages of preimplantation development, as well as genes uniquely expressed at each of these developmental stages, is expected to be of used in future experiments, potentially providing important information regarding sample quality. More generally, alterations in the level of expression of the microRNA processing genes, that play a significant role during development and have an impact in the expression of other genes, might also be used as an indicator of oocyte and embryo quality. Additionally, this study identified genes which may show a stable expression between human oocyte and blastocyst stage and may thus be candidate ‘housekeeping’ genes. Further work on validation of these results is important, especially as it has recently been recommended that more than one reference genes should be used for accurate normalization and interpretation of the expression results (Vandesompele et al., 2002). Finally, the DM1-associated genes detected in the oocyte and embryo samples

could form the focus of further investigations for the comparison of gene expression between DM1-affected and unaffected samples.

4.4 Future work

The results presented in this study have provided the basis for future experiments to expand on current knowledge of the mechanism and pathogenesis of DM1. The availability of the new protocols will increase the number of patients with DM1 that can undergo PGD. The continuing PGD work, combined with analysis of data from diagnosis and follow-up analysis of embryos unsuitable for transfer, may provide a clearer picture regarding transmission of different sized repeat alleles, the mechanisms involved in repeat transmission as well as affected vs. unaffected embryo development.

The results from this study have also raised several questions that could be addressed in future work. Interesting findings and observations have included abnormally fertilised oocytes, poor quality embryos and identification of chromosomally abnormal embryos from patients with DM1. The possible incidence of monosomy 19 could not be confirmed. The co-amplification of several markers on chromosome 19 may be of use in identifying cases of monosomy, while testing of markers on different chromosomes may suggest cases of haploid embryos. However, such an approach would be very difficult at the single-cell level. For that purpose, chromosomal analysis of embryos from patients with DM1 and comparison with results from other single gene PGD cases has already been initiated in our department. It remains to be seen whether a combination of chromosomal and molecular analysis of the preimplantation embryo will be of benefit for patients with DM1, in order to enable the identification of embryos more likely to implant. This analysis has been previously attempted by combining WGA followed by F-PCR and CGH analysis, and may, in the future, be possible by genetic analysis of single biopsied cells by DNA microarray technology (Kuliev and Verlinsky, 2008).

Follow-up analysis of spare embryos following PGD may also be extended by optimising a new protocol at the single cell level, to co-amplify the AMLXY primer along with the other markers of the DM1 triplex or mTP-PCR protocols. The aim of this would be to establish whether there is an association between repeat size, embryo sex and/or embryo development. In this study this analysis was initiated on only a few embryos following amplification by MDA. Since these results indicated a very high ADO rate, it is expected that a more accurate and faster at the same time, analysis will be possible by simple F-PCR. Because of the small product size of the AMXY primer, it

is anticipated that its amplification on single cells and co-amplification along with the other markers in a multiplex PCR will not require extensive optimisation.

This study also included testing of buccal cells from two babies born following PGD for two families. The continuing analysis may provide interesting results regarding the characteristics and grade of implanting embryos. Additionally, embryo rebiopsy has not been previously described by another PGD centre; although it increases the rate of embryo diagnosis further evidence is necessary to investigate the possible impact of rebiopsy on embryo development and implantation.

Finally, during the course of this study several immature oocytes were collected from patients with DM1 and stored at -80°C (section 2.3.2.2). These were destined to be used, following validation of the TP-PCR protocol, for estimation of the repeat size. The aim was to obtain some indication regarding the timing of the repeat expansion, at the same time focus on a more patient-specific analysis concerning the size of the expansion and associate oocyte analysis with remaining embryo diagnosis. Because of the inaccuracy of sizing by TP-PCR, as found in this study, these oocytes were not processed and may be used instead for repeat sizing by Southern blotting, as has been previously reported (De Temmerman et al., 2004). Further work for this may be, however, necessary as protocols for performing Southern blotting from a single cell have not as yet become available.

The results from the microarray analysis have provided important information to guide further experiments by real-time PCR technology. The identification of genes that may play a significant role at the oocyte and blastocyst stages of preimplantation development, as well as genes that may indicate good oocyte and embryo quality, will be of use in future expression studies. The identified list of housekeeping genes with apparently similar expression between the two stages indicates which genes to further target for investigation by real-time PCR.

One of the difficulties encountered in this study, due to the limited number of DM1 PGD cases at the time, was the collection of DM1 affected oocytes and embryos. Hopefully, the collection of more of these samples in the future will enable a comparison of the normal to the disease state, using the information regarding the

expression level of relevant genes, as provided by the microarray data and possibly focusing on transcription factors and microRNA pathway genes. Affected and unaffected immature oocytes may be identified by isolation of the oocyte polar bodies and testing each PB using the mTP-PCR protocol. The suggested oocyte genotype may also be confirmed by simultaneous DNA/RNA isolation of the oocyte and testing of the DNA using mTP-PCR. Expression studies on the affected and unaffected oocytes may indicate additional differences in chromosomal constitution or expression.

In summary, future work stemming from this study may involve accumulation of data from PGD and follow-up analysis, including development of a new mTP-PCR with AMXY protocol, chromosomal analysis of embryos from patients with DM1 and comparison with other monogenic disorders, repeat sizing by Southern blotting and real-time PCR analysis. Understanding the molecular events of early preimplantation development and investigating the changes that may be associated with the presence of the CTG repeat expansion may provide a further insight into the mechanisms controlling DM1 development and progression; genes that play a role in the expansion pathway may become future targets for therapeutic intervention not only in DM1 but also other repeat expansion disorders.

5. Reference list

2004. The Preimplantation Genetic Diagnosis International Society (PGDIS): Guidelines for good practice in PGD. *Reprod. Biomed. Online.* 9: 430-434.

Abbott,A.L., R.A.Fissore, and T.Ducibella. 1999. Incompetence of preovulatory mouse oocytes to undergo cortical granule exocytosis following induced calcium oscillations. *Dev. Biol.* 207: 38-48.

Abbruzzese,C., P.S.Costanzi, B.Mariani, F.K.Gould, J.P.McAbney, D.G.Monckton, T.Ashizawa, and M.Giacanelli. 2002. Instability of a premutation allele in homozygous patients with myotonic dystrophy type 1. *Ann. Neurol.* 52: 435-441.

Abe,K. 2002. Somatic instability of CTG expansion in cancer tissue? *Intern. Med.* 41: 253.

Abou-Sleiman,P.M., A.Apessos, J.C.Harper, P.Serhal, and J.D.Delhanty. 2002. Pregnancy following preimplantation genetic diagnosis for Crouzon syndrome. *Mol. Hum. Reprod.* 8: 304-309.

Acton,R.T., C.A.Rivers, B.Watson, and S.J.Oh. 2007. DMPK-associated myotonic dystrophy and CTG repeats in Alabama African Americans. *Clin. Genet.* 72: 448-453.

Adjaye,J., R.Daniels, V.Bolton, and M.Monk. 1997. cDNA libraries from single human preimplantation embryos. *Genomics* 46: 337-344.

Adjaye,J., J.Huntriss, R.Herwig, A.BenKahla, T.C.Brink, C.Wierling, C.Hultschig, D.Groth, M.L.Yaspo, H.M.Picton, R.G.Gosden, and H.Lehrach. 2005. Primary differentiation in the human blastocyst: comparative molecular portraits of inner cell mass and trophectoderm cells. *Stem Cells* 23: 1514-1525.

Alfadhli,S., A.E.Elshafey, L.Bastaki, and S.Al-Awadi. 2004. CTG repeat number at the myotonic dystrophy locus in healthy Kuwaiti individuals: possible explanation of why myotonic dystrophy is rare in Kuwait. *Arch. Neurol.* 61: 895-898.

Allison,D.B., X.Cui, G.P.Page, and M.Sabripour. 2006. Microarray data analysis: from disarray to consolidation and consensus. *Nat. Rev. Genet.* 7: 55-65.

Amicucci,P., M.Gennarelli, G.Novelli, and B.Dallapiccola. 2000. Prenatal diagnosis of myotonic dystrophy using fetal DNA obtained from maternal plasma. *Clin. Chem.* 46: 301-302.

Amiel,J., V.Raclin, J.M.Jouannic, N.Morichon, H.Hoffman-Radvanyi, M.Dommergues, J.Feingold, A.Munnich, and J.P.Bonnefont. 2001. Trinucleotide repeat contraction: a pitfall in prenatal diagnosis of myotonic dystrophy. *J. Med. Genet.* 38: 850-852.

Andreu-Vieyra,C., Y.N.Lin, and M.M.Matzuk. 2006. Mining the oocyte transcriptome. *Trends Endocrinol. Metab* 17: 136-143.

Antonini,G., F.Soscia, F.Giubilei, C.A.De, F.Gragnani, S.Morino, A.Ruberto, and R.Tatarelli. 2006. Health-related quality of life in myotonic dystrophy type 1 and its relationship with cognitive and emotional functioning. *J. Rehabil. Med.* 38: 181-185.

Ao,A., D.Wells, A.H.Handyside, R.M.Winston, and J.D.Delhanty. 1998. Preimplantation genetic diagnosis of inherited cancer: familial adenomatous polyposis coli. *J. Assist. Reprod. Genet.* 15: 140-144.

Asano,A., N.Motomura, S.Yokota, H.Yoneda, T.Sakai, and S.Tsutsumi. 2000. Myotonic dystrophy associated with 47 XYY syndrome. *Psychiatry Clin. Neurosci.* 54: 113-116.

Ashizawa,T., M.Anvret, M.Baiget, J.M.Barcelo, H.Brunner, A.M.Cobo, B.Dallapiccola, R.G.Fenwick, Jr., U.Grandell, H.Harley, et al. 1994. Characteristics of intergenerational contractions of the CTG repeat in myotonic dystrophy. *Am. J. Hum. Genet.* 54: 414-423.

Ashley,A.E. and S.L.Sherman. 1995. Population dynamics of a meiotic/mitotic expansion model for the fragile X syndrome. *Am. J. Hum. Genet.* 57: 1414-1425.

Assou,S., T.Anahory, V.Pantesco, T.Le Carrour, F.Pellestor, B.Klein, L.Reyftmann, H.Dechaud, J.De Vos, and S.Hamamah. 2006. The human cumulus--oocyte complex gene-expression profile. *Hum. Reprod.* 21: 1705-1719.

Assou,S., D.Haouzi, K.Mahmoud, A.Aouacheria, Y.Guillemin, V.Pantesco, T.Reme, H.Dechaud, V.J.De, and S.Hamamah. 2008. A non-invasive test for assessing embryo potential by gene expression profiles of human cumulus cells: a proof of concept study. *Mol. Hum. Reprod.*14 (12): 711-9

Baart,E.B., E.Martini, M.J.Eijkemans, O.D.Van, N.G.Beckers, A.Verhoeff, N.S.Macklon, and B.C.Fauser. 2007a. Milder ovarian stimulation for in-vitro fertilization reduces aneuploidy in the human preimplantation embryo: a randomized controlled trial. *Hum. Reprod.* 22: 980-988.

Baart,E.B., d.B.van, I, E.Martini, H.J.Eussen, B.C.Fauser, and O.D.Van. 2007b. FISH analysis of 15 chromosomes in human day 4 and 5 preimplantation embryos: the added value of extended aneuploidy detection. *Prenat. Diagn.* 27: 55-63.

Balakier,H., D.Bouman, A.Sojecki, C.Librach, and J.A.Squire. 2002. Morphological and cytogenetic analysis of human giant oocytes and giant embryos. *Hum. Reprod.* 17: 2394-2401.

Baldi,P., S.Brunak, Y.Chauvin, and A.G.Pedersen. 1999. Structural basis for triplet repeat disorders: a computational analysis. *Bioinformatics.* 15: 918-929.

Banerjee,I., M.Shevlin, M.Taranissi, A.Thornhill, H.Abdalla, O.Ozturk, J.Barnes, and A.Sutcliffe. 2008. Health of children conceived after preimplantation genetic diagnosis: a preliminary outcome study. *Reprod. Biomed. Online.* 16: 376-381.

Bartel,D.P. 2004. MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell* 116: 281-297.

Bates,G.P. and R.Gonitel. 2006. Mouse models of triplet repeat diseases. *Mol. Biotechnol.* 32: 147-158.

Bell,C.E., M.D.Calder, and A.J.Watson. 2008. Genomic RNA Profiling and the Program Controlling Preimplantation Mammalian Development. *Mol. Hum. Reprod* 14: 691-701.

Bermudez,M.G., W.Piyamongkol, S.Tomaz, E.Dudman, J.K.Sherlock, and D.Wells. 2003. Single-cell sequencing and mini-sequencing for preimplantation genetic diagnosis. *Prenat. Diagn.* 23: 669-677.

Bermudez,M.G., D.Wells, H.Malter, S.Munne, J.Cohen, and N.M.Steuerwald. 2004. Expression profiles of individual human oocytes using microarray technology. *Reprod. Biomed. Online.* 8: 325-337.

Berul,C.I., C.T.Maguire, M.J.Aronovitz, J.Greenwood, C.Miller, J.Gehrmann, D.Housman, M.E.Mendelsohn, and S.Reddy. 1999. DMPK dosage alterations result in atrioventricular conduction abnormalities in a mouse myotonic dystrophy model. *J. Clin. Invest* 103: R1-R7.

Blake,D.A., C.M.Farquhar, N.Johnson, and M.Proctor. 2007. Cleavage stage versus blastocyst stage embryo transfer in assisted conception. *Cochrane. Database. Syst. Rev.* CD002118.

Boada,M., M.Carrera, L.De, I, M.Sandalinas, P.N.Barri, and A.Veiga. 1998. Successful use of a laser for human embryo biopsy in preimplantation genetic diagnosis: report of two cases. *J. Assist. Reprod. Genet.* 15: 302-307.

Borkenhagen,A., E.Brahler, S.Wisch, Y.Stobel-Richter, B.Strauss, and H.Kentenich. 2007. Attitudes of German infertile couples towards preimplantation genetic diagnosis for different uses: a comparison to international studies. *Hum. Reprod.* 22: 2051-2057.

Botta,A., F.Rinaldi, C.Catalli, L.Vergani, E.Bonifazi, V.Romeo, E.Loro, A.Viola, C.Angelini, and G.Novelli. 2008. The CTG repeat expansion size correlates with the splicing defects observed in muscles from myotonic dystrophy type 1 patients. *J. Med. Genet.* 45: 639-646.

Boyum,A. 1968. Isolation of mononuclear cells and granulocytes from human blood. Isolation of monuclear cells by one centrifugation, and of granulocytes by combining centrifugation and sedimentation at 1 g. *Scand. J. Clin. Lab Invest Suppl* 97: 77-89.

Braude,P., V.Bolton, and S.Moore. 1988. Human gene expression first occurs between the four- and eight-cell stages of preimplantation development. *Nature* 332: 459-461.

Brook,J.D., M.E.McCurrach, H.G.Harley, A.J.Buckler, D.Church, H.Aburatani, K.Hunter, V.P.Stanton, J.P.Thirion, T.Hudson, *et al*. 1992. Molecular basis of myotonic dystrophy: expansion of a trinucleotide (CTG) repeat at the 3' end of a transcript encoding a protein kinase family member. *Cell* 68: 799-808.

Buccione,R., B.C.Vanderhyden, P.J.Caron, and J.J.Eppig. 1990. FSH-induced expansion of the mouse cumulus oophorus in vitro is dependent upon a specific factor(s) secreted by the oocyte. *Dev. Biol.* 138: 16-25.

Burlet,P., N.Frydman, N.Gigarel, V.Kerbrat, G.Tachdjian, E.Feyereisen, J.P.Bonnefont, R.Frydman, A.Munnich, and J.Steffann. 2006. Multiple displacement amplification improves PGD for fragile X syndrome. *Mol. Hum. Reprod.* 12: 647-652.

Callahan,J.L., K.J.Andrews, V.A.Zakian, and C.H.Freudenreich. 2003. Mutations in yeast replication proteins that increase CAG/CTG expansions also increase repeat fragility. *Mol. Cell Biol.* 23: 7849-7860.

Cao,H., C.S.Yang, and T.M.Rana. 2008. Evolutionary emergence of microRNAs in human embryonic stem cells. *PLoS. ONE.* 3: e2820.

Carey,N., K.Johnson, P.Nokelainen, L.Peltonen, M.L.Savontaus, V.Juvonen, M.Anvret, U.Grandell, K.Chotai, E.Robertson, and . 1994. Meiotic drive at the myotonic dystrophy locus? *Nat. Genet.* 6: 117-118.

Casella,M., M.Lucarelli, M.Simili, P.Beffy, R.Del Carratore, F.Minichilli, C.Chisari, and S.Simi. 2003. Spontaneous chromosome loss and colcemid resistance in lymphocytes from patients with myotonic dystrophy type 1. *Cytogenet. Genome Res.* 100: 224-229.

Cauffman,G., d.Van, V, I.Liebaers, and A.Van Steirteghem. 2005. Oct-4 mRNA and protein expression during human preimplantation development. *Mol. Hum. Reprod* .11(3): 173-81

Chakraborty,R., D.N.Stivers, R.Deka, L.M.Yu, M.D.Shriver, and R.E.Ferrell. 1996. Segregation distortion of the CTG repeats at the myotonic dystrophy locus. *Am. J. Hum. Genet.* 59: 109-118.

Chatzimeletiou,K., E.E.Morrison, N.Prapas, Y.Prapas, and A.H.Handyside. 2005. Spindle abnormalities in normally developing and arrested human preimplantation embryos in vitro identified by confocal laser scanning microscopy. *Hum. Reprod.* 20: 672-682.

Cho,D.H., C.P.Thienes, S.E.Mahoney, E.Analau, G.N.Filippova, and S.J.Tapscott. 2005. Antisense transcription and heterochromatin at the DM1 CTG repeats are constrained by CTCF. *Mol. Cell* 20: 483-489.

Cillo,F., T.A.Brevini, S.Antonini, A.Paffoni, G.Ragni, and F.Gandolfi. 2007. Association between human oocyte developmental competence and expression levels of some cumulus genes. *Reproduction.* 134: 645-650.

Cleary,J.D. and C.E.Pearson. 2003. The contribution of cis-elements to disease-associated repeat instability: clinical and experimental evidence. *Cytogenet. Genome Res.* 100: 25-55.

Cohen,J., D.Wells, and S.Munne. 2007. Removal of 2 cells from cleavage stage embryos is likely to reduce the efficacy of chromosomal tests that are used to enhance implantation rates. *Fertil. Steril.* 87: 496-503.

Corn,C.M., C.Hauser-Kronberger, M.Moser, G.Tews, and T.Ebner. 2005. Predictive value of cumulus cell apoptosis with regard to blastocyst development of corresponding gametes. *Fertil. Steril.* 84: 627-633.

Cui,X.F., H.H.Li, T.M.Goradia, K.Lange, H.H.Kazazian, Jr., D.Galas, and N.Arnheim. 1989. Single-sperm typing: determination of genetic distance between the G gamma-globin and parathyroid hormone loci by using the polymerase chain reaction and allele-specific oligomers. *Proc. Natl. Acad. Sci. U. S. A* 86: 9389-9393.

Cui,X.S., X.H.Shen, and N.H.Kim. 2007. Dicer1 expression in preimplantation mouse embryos: Involvement of Oct3/4 transcription at the blastocyst stage. *Biochem. Biophys. Res. Commun.* 352: 231-236.

Culjkovic,B., O.Stojkovic, S.Vukosavic, D.Savic, V.Rakocevic, S.Apostolski, and S.Romac. 2002. CTG repeat polymorphism in DMPK gene in healthy Yugoslav population. *Acta Neurol. Scand.* 105: 55-58.

Cummings,C.J. and H.Y.Zoghbi. 2000. Fourteen and counting: unraveling trinucleotide repeat diseases. *Hum. Mol. Genet.* 9: 909-916.

Daniels,R., T.Kinis, P.Serhal, and M.Monk. 1995. Expression of the myotonin protein kinase gene in preimplantation human embryos. *Hum. Mol. Genet.* 4: 389-393.

Dansithong,W., S.Paul, L.Comai, and S.Reddy. 2005. MBNL1 is the primary determinant of focus formation and aberrant insulin receptor splicing in DM1. *J. Biol. Chem.* 280: 5773-5780.

Day,J.W., K.Ricker, J.F.Jacobsen, L.J.Rasmussen, K.A.Dick, W.Kress, C.Schneider, M.C.Koch, G.J.Beilman, A.R.Harrison, J.C.Dalton, and L.P.Ranum. 2003. Myotonic dystrophy type 2: molecular, diagnostic and clinical spectrum. *Neurology* 60: 657-664.

De Temmerman,N., K.Sermon, S.Seneca, M.De Rycke, P.Hilven, W.Lissens, A.Van Steirteghem, and I.Liebaers. 2004. Intergenerational instability of the expanded CTG repeat in the DMPK gene: studies in human gametes and preimplantation embryos. *Am. J. Hum. Genet.* 75: 325-329.

De Vos,A. and A.Van Steirteghem. 2001. Aspects of biopsy procedures prior to preimplantation genetic diagnosis. *Prenat. Diagn.* 21: 767-780.

Dean,N.L., J.C.Loredo-Osti, T.M.Fujiwara, K.Morgan, S.L.Tan, A.K.Naumova, and A.Ao. 2006a. Transmission ratio distortion in the myotonic dystrophy locus in human preimplantation embryos. *Eur. J. Hum. Genet.* 14: 299-306.

Dean,N.L., S.L.Tan, and A.Ao. 2001. The development of preimplantation genetic diagnosis for myotonic dystrophy using multiplex fluorescent polymerase chain reaction and its clinical application. *Mol. Hum. Reprod.* 7: 895-901.

Dean,N.L., S.L.Tan, and A.Ao. 2006b. Instability in the transmission of the myotonic dystrophy CTG repeat in human oocytes and preimplantation embryos. *Fertil. Steril.* 86: 98-105.

Deka,R., P.P.Majumder, M.D.Shriver, D.N.Stivers, Y.Zhong, L.M.Yu, R.Barrantes, S.J.Yin, T.Miki, J.Hundrieser, C.H.Bunker, S.T.McGarvey, S.Sakallah, R.E.Ferrell, and R.Chakraborty. 1996. Distribution and evolution of CTG repeats at the myotonin protein kinase gene in human populations. *Genome Res.* 6: 142-154.

Dekel,N. and W.H.Beers. 1980. Development of the rat oocyte in vitro: inhibition and induction of maturation in the presence or absence of the cumulus oophorus. *Dev. Biol.* 75: 247-254.

Delhanty,J. 2006. What are the issues surrounding preimplantation genetic diagnosis for late-onset disorders? *Expert. Rev. Mol. Diagn.* 6: 5-6.

Delhanty,J.D., A.H.Handyside, and R.M.Winston. 1994. Preimplantation diagnosis. *Lancet* 343: 1569-1570.

Delhanty,J.D., J.C.Harper, A.Ao, A.H.Handyside, and R.M.Winston. 1997. Multicolour FISH detects frequent chromosomal mosaicism and chaotic division in normal preimplantation embryos from fertile patients. *Hum. Genet.* 99: 755-760.

Delhanty,J.D. and D.Wells. 2002. Preimplantation genetic diagnosis: an alternative to prenatal diagnosis. *Expert. Rev. Mol. Diagn.* 2: 395-399.

Dell'Aquila,M.E., T.De Santis, Y.S.Cho, S.J.Reshkin, A.M.Caroli, F.Maritato, P.Minoia, and V.Casavola. 2006. Localization and quantitative expression of the calcium-sensing receptor protein in human oocytes. *Fertil. Steril.* 85 Suppl 1: 1240-1247.

Dhanjal,S., G.Kakourou, T.Mamas, N.Saleh, A.Doshi, S.Gotts, S.Nuttall, K.Fordham, P.Serhal, J.Delhanty, J.Harper, and S.Sengupta. 2007. Preimplantation genetic diagnosis for retinoblastoma predisposition. *Br. J. Ophthalmol.* 91: 1090-1091.

Die-Smulders,C.E., H.J.Smeets, W.Loots, H.B.Anten, J.F.Mirandolle, J.P.Geraedts, and C.J.Howeler. 1997. Paternal transmission of congenital myotonic dystrophy. *J. Med. Genet.* 34: 930-933.

Dobson,A.T., R.Raja, M.J.Abeyta, T.Taylor, S.Shen, C.Haqq, and R.A.Pera. 2004. The unique transcriptome through day 3 of human preimplantation development. *Hum. Mol. Genet.* 13: 1461-1470.

Drasinover,V., S.Ehrlich, N.Magal, E.Taub, V.Libman, T.Shohat, G.J.Halpern, and M.Shohat. 2000. Increased transmission of intermediate alleles of the FMR1 gene compared with normal alleles among female heterozygotes. *Am. J. Med. Genet.* 93: 155-157.

Dreesen,J., M.Drusedau, H.Smeets, C.de Die-Smulders, E.Coonen, J.Dumoulin, M.Gielen, J.Evers, J.Herbergs, and J.Geraedts. 2008. Validation of Preimplantation Genetic Diagnosis by PCR analysis: genotype comparison of the blastomere and corresponding embryo, implications for clinical practice. *Mol. Hum. Reprod.*14:573-579.

Duranthon,V., A.J.Watson, and P.Lonergan. 2008. Preimplantation embryo programming: transcription, epigenetics, and culture environment. *Reproduction.* 135: 141-150.

Ebralidze,A., Y.Wang, V.Petkova, K.Ebralidse, and R.P.Junghans. 2004. RNA leaching of transcription factors disrupts transcription in myotonic dystrophy. *Science* 303: 383-387.

Edstrom,L. 1999. Dystrophia myotonica. Clinical, pathophysiological and molecular aspects. *Scand. J. Rehabil. Med. Suppl* 39: 47-52.

el Hashemite,N. and J.D.Delhanty. 1997. A technique for eliminating allele specific amplification failure during DNA amplification of heterozygous cells for preimplantation diagnosis. *Mol. Hum. Reprod.* 3: 975-978.

Emiliani,S., E.Gonzalez-Merino, Y.Englert, and M.Abramowicz. 2004. Comparison of the validity of preimplantation genetic diagnosis for embryo chromosomal anomalies by fluorescence in situ hybridization on one or two blastomeres. *Genet. Test.* 8: 69-72.

Evans,K., A.Fryer, C.Inglehearn, J.Duvall-Young, J.L.Whittaker, C.Y.Gregory, R.Butler, N.Ebenezer, D.M.Hunt, and S.Bhattacharya. 1994. Genetic linkage of cone-rod retinal dystrophy to chromosome 19q and evidence for segregation distortion. *Nat. Genet.* 6: 210-213.

Everett,C.M. and N.W.Wood. 2004. Trinucleotide repeats and neurodegenerative disease. *Brain* 127: 2385-2405.

Falco,G., I.Stanghellini, and M.S.Ko. 2006. Use of Chuk as an internal standard suitable for quantitative RT-PCR in mouse preimplantation embryos. *Reprod. Biomed. Online.* 13: 394-403.

Falk,M., M.Vojtiskova, Z.Lukas, I.Kroupova, and U.Froster. 2006. Simple procedure for automatic detection of unstable alleles in the myotonic dystrophy and Huntington's disease loci. *Genet. Test.* 10: 85-97.

Fardaei,M., K.Larkin, J.D.Brook, and M.G.Hamshere. 2001. In vivo co-localisation of MBNL protein with DMPK expanded-repeat transcripts. *Nucleic Acids Res.* 29: 2766-2771.

Feenan,K. and M.Herbert. 2006. Can 'abnormally' fertilized zygotes give rise to viable embryos? *Hum. Fertil. (Camb.)* 9: 157-169.

Feyereisen,E., A.Amar, V.Kerbrat, J.Steffann, A.Munnich, M.Vekemans, R.Frydman, and N.Frydman. 2006. Myotonic dystrophy: does it affect ovarian follicular status and responsiveness to controlled ovarian stimulation? *Hum. Reprod.* 21: 175-182.

Feyereisen,E., J.Steffann, S.Romana, M.Lelorc'h, P.Ray, V.Kerbrat, G.Tachdjian, R.Frydman, and N.Frydman. 2007. Five years' experience of preimplantation genetic diagnosis in the Parisian Center: outcome of the first 441 started cycles. *Fertil. Steril.* 87: 60-73.

Filippova,G.N., C.P.Thienes, B.H.Penn, D.H.Cho, Y.J.Hu, J.M.Moore, T.R.Klesert, V.V.Lobanenkov, and S.J.Tapscott. 2001. CTCF-binding sites flank CTG/CAG repeats and form a methylation-sensitive insulator at the DM1 locus. *Nat. Genet.* 28: 335-343.

Findlay,I., P.Ray, P.Quirke, A.Rutherford, and R.Lilford. 1995. Allelic drop-out and preferential amplification in single cells and human blastomeres: implications for preimplantation diagnosis of sex and cystic fibrosis. *Hum. Reprod.* 10: 1609-1618.

Fiorentino,F., A.Biricik, H.Karadayi, H.Berkil, G.Karlikaya, S.Sertyel, D.Podini, M.Baldi, M.C.Magli, L.Gianaroli, and S.Kahraman. 2004. Development and clinical application of a strategy for preimplantation genetic diagnosis of single gene disorders combined with HLA matching. *Mol. Hum. Reprod.* 10: 445-460.

Fiorentino,F., A.Biricik, A.Nuccitelli, P.R.De, S.Kahraman, M.Iacobelli, V.Trengia, D.Caserta, M.A.Bonu, A.Borini, and M.Baldi. 2006. Strategies and clinical outcome of 250 cycles of Preimplantation Genetic Diagnosis for single gene disorders. *Hum. Reprod.* 21: 670-684.

Fiorentino,F., M.C.Magli, D.Podini, A.P.Ferraretti, A.Nuccitelli, N.Vitale, M.Baldi, and L.Gianaroli. 2003. The minisequencing method: an alternative strategy for preimplantation genetic diagnosis of single gene disorders. *Mol. Hum. Reprod.* 9: 399-410.

Florencia,G., S.Irene, and F.Veronica. 2006. Fragile-X mental retardation: molecular diagnosis in Argentine patients. *J. Biochem. Mol. Biol.* 39: 766-773.

Fokstuen,S., J.Myring, C.Evans, and P.S.Harper. 2001. Presymptomatic testing in myotonic dystrophy: genetic counselling approaches. *J. Med. Genet.* 38: 846-850.

Fragouli,E. 2007. Preimplantation genetic diagnosis: present and future. *J. Assist. Reprod. Genet.* 24: 201-207.

Fragouli,E., M.Lenzi, R.Ross, M.Katz-Jaffe, W.B.Schoolcraft, and D.Wells. 2008. Comprehensive molecular cytogenetic analysis of the human blastocyst stage. *Hum. Reprod.* 23: 2596-2608.

Fragouli,E., D.Wells, A.Thornhill, P.Serhal, M.J.Faed, J.C.Harper, and J.D.Delhanty. 2006a. Comparative genomic hybridization analysis of human oocytes and polar bodies. *Hum. Reprod.* 21: 2319-2328.

Fragouli,E., D.Wells, K.M.Whalley, J.A.Mills, M.J.Faed, and J.D.Delhanty. 2006b. Increased susceptibility to maternal aneuploidy demonstrated by comparative genomic hybridization analysis of human MII oocytes and first polar bodies. *Cytogenet. Genome Res.* 114: 30-38.

Francke,U., M.S.Brown, and J.L.Goldstein. 1984. Assignment of the human gene for the low density lipoprotein receptor to chromosome 19: synteny of a receptor, a ligand, and a genetic disease. *Proc. Natl. Acad. Sci. U. S. A* 81: 2826-2830.

Fu,Y.H., A.Pizzuti, R.G.Fenwick, Jr., J.King, S.Rajnarayan, P.W.Dunne, J.Dubel, G.A.Nasser, T.Ashizawa, P.de Jong, *et al* . 1992. An unstable triplet repeat in a gene related to myotonic muscular dystrophy. *Science* 255: 1256-1258.

Fulka,H., M.Mrazek, O.Tepla, and J.Fulka, Jr. 2004. DNA methylation pattern in human zygotes and developing embryos. *Reproduction.* 128: 703-708.

Gardner,D.K., W.B.Schoolcraft, L.Wagley, T.Schlenker, J.Stevens, and J.Hesla. 1998a. A prospective randomized trial of blastocyst culture and transfer in in-vitro fertilization. *Hum. Reprod.* 13: 3434-3440.

Gardner,D.K., P.Vella, M.Lane, L.Wagley, T.Schlenker, and W.B.Schoolcraft. 1998b. Culture and transfer of human blastocysts increases implantation rates and reduces the need for multiple embryo transfers. *Fertil. Steril.* 69: 84-88.

Gatchel,J.R. and H.Y.Zoghbi. 2005. Diseases of unstable repeat expansion: mechanisms and common principles. *Nat. Rev. Genet.* 6: 743-755.

Gennarelli,M., B.Dallapiccola, M.Baiget, L.Martorell, and G.Novelli. 1994. Meiotic drive at the myotonic dystrophy locus. *J. Med. Genet.* 31: 980.

Gigarel,N., N.Frydman, P.Burlet, V.Kerbrat, J.Steffann, R.Frydman, A.Munnich, and P.F.Ray. 2004. Single cell co-amplification of polymorphic markers for the indirect preimplantation genetic diagnosis of hemophilia A, X-linked adrenoleukodystrophy, X-linked hydrocephalus and incontinentia pigmenti loci on Xq28. *Hum. Genet.* 114: 298-305.

Girardet,A., M.S.McPeek, E.P.Leeflang, F.Munier, N.Arnheim, M.Claustres, and F.Pellestor. 2000. Meiotic segregation analysis of RB1 alleles in retinoblastoma pedigrees by use of single-sperm typing. *Am. J. Hum. Genet.* 66: 167-175.

Gitlin,S.A., S.E.Lanzendorf, and W.E.Gibbons. 1996. Polymerase chain reaction amplification specificity: incidence of allele dropout using different DNA preparation methods for heterozygous single cells. *J. Assist. Reprod. Genet.* 13: 107-111.

Gomes-Pereira,M., L.Foiry, A.Nicole, A.Huguet, C.Junien, A.Munnich, and G.Gourdon. 2007. CTG trinucleotide repeat "big jumps": large expansions, small mice. *PLoS. Genet.* 3: e52.

Gomes-Pereira,M., M.T.Fortune, L.Ingram, J.P.McAbney, and D.G.Monckton. 2004. Pms2 is a genetic enhancer of trinucleotide CAG.CTG repeat somatic mosaicism: implications for the mechanism of triplet repeat expansion. *Hum. Mol. Genet.* 13: 1815-1825.

Goossens,V., R.M.De, V.A.De, C.Staessen, A.Michiels, W.Verpoest, S.A.Van, C.Bertrand, I.Liebaers, P.Devroey, and K.Sermon. 2008a. Diagnostic efficiency, embryonic development and clinical outcome after the biopsy of one or two blastomeres for preimplantation genetic diagnosis. *Hum. Reprod.* 23: 481-492.

Goossens,V., G.Harton, C.Moutou, P.N.Scriven, J.Traeger-Synodinos, K.Sermon, and J.C.Harper. 2008b. ESHRE PGD Consortium data collection VIII: cycles from January to December 2005 with pregnancy follow-up to October 2006. *Hum. Reprod.* 23: 2629-2645.

Grace,J., T.El Toukhy, P.Scriven, C.Ogilvie, S.Pickering, A.Lashwood, F.Flinter, Y.Khalaf, and P.Braude. 2006. Three hundred and thirty cycles of preimplantation genetic diagnosis for serious genetic disease: clinical considerations affecting outcome. *BJOG.* 113: 1393-1401.

Griffin,D.K., L.J.Wilton, A.H.Handyside, R.M.Winston, and J.D.Delhanty. 1992. Dual fluorescent in situ hybridisation for simultaneous detection of X and Y chromosome-specific probes for the sexing of human preimplantation embryonic nuclei. *Hum. Genet.* 89: 18-22.

Guiraud-Dogan,C., A.Huguet, M.Gomes-Pereira, E.Brisson, G.Bassez, C.Junien, and G.Gourdon. 2007. DM1 CTG expansions affect insulin receptor isoforms expression in various tissues of transgenic mice. *Biochim. Biophys. Acta* 1772: 1183-1191.

Gutierrez-Mateo,C., J.F.Sanchez-Garcia, J.Fischer, S.Tormasi, J.Cohen, S.Munne, and D.Wells. 2008. Preimplantation genetic diagnosis of single-gene disorders: experience with more than 200 cycles conducted by a reference laboratory in the United States. *Fertil. Steril.*[Epub ahead of print].

Hamatani,T., M.S.Ko, M.Yamada, N.Kuji, Y.Mizusawa, M.Shoji, T.Hada, H.Asada, T.Maruyama, and Y.Yoshimura. 2006. Global gene expression profiling of preimplantation embryos. *Hum. Cell* 19: 98-117.

Hamshere,M.G., H.Harley, P.Harper, J.D.Brook, and J.F.Brookfield. 1999. Myotonic dystrophy: the correlation of (CTG) repeat length in leucocytes with age at onset is significant only for patients with small expansions. *J. Med. Genet.* 36: 59-61.

Handyside,A.H., E.H.Kontogianni, K.Hardy, and R.M.Winston. 1990. Pregnancies from biopsied human preimplantation embryos sexed by Y-specific DNA amplification. *Nature* 344: 768-770.

Handyside,A.H., J.G.Lesko, J.J.Tarin, R.M.Winston, and M.R.Hughes. 1992. Birth of a normal girl after in vitro fertilization and preimplantation diagnostic testing for cystic fibrosis. *N. Engl. J. Med.* 327: 905-909.

Hao,M., K.Akrami, K.Wei, C.De Diego, N.Che, J.H.Ku, J.Tidball, M.C.Graves, P.B.Shieh, and F.Chen. 2008. Muscleblind-like 2 (Mbnl2) -deficient mice as a model for myotonic dystrophy. *Dev. Dyn.* 237: 403-410.

Hardy,K., K.L.Martin, H.J.Leese, R.M.Winston, and A.H.Handyside. 1990. Human preimplantation development in vitro is not adversely affected by biopsy at the 8-cell stage. *Hum. Reprod.* 5: 708-714.

Harper,J.C. and A.Thornhill. 2001. Embryo biopsy. in *Preimplantation Genetic Diagnosis* (ed. J.C.Harper, J.D.Delhanty, and A.Handyside), John Wiley & Sons, LTD, UK.

Harper,J.C. and D.Wells. 1999. Recent advances and future developments in PGD. *Prenat. Diagn.* 19: 1193-1199.

Harper,J.C., D.Wells, W.Piyamongkol, P.Abou-Sleiman, A.Apessos, A.Ioulianos, M.Davis, A.Doshi, P.Serhal, M.Ranieri, C.Rodeck, and J.D.Delhanty. 2002. Preimplantation genetic diagnosis for single gene disorders: experience with five single gene disorders. *Prenat. Diagn.* 22: 525-533.

Hartshorn,C., J.J.Eckert, O.Hartung, and L.J.Wangh. 2007. Single-cell duplex RT-LATE-PCR reveals Oct4 and Xist RNA gradients in 8-cell embryos. *BMC. Biotechnol.* 7: 87.

Heidenfelder,B.L., A.M.Makhov, and M.D.Topal. 2003. Hairpin formation in Friedreich's ataxia triplet repeat expansion. *J. Biol. Chem.* 278: 2425-2431.

Hellani,A., K.bu-Amero, J.Azouri, and S.El-Akoum. 2008. Successful pregnancies after application of array-comparative genomic hybridization in PGS-aneuploidy screening. *Reprod. Biomed. Online.* 17: 841-847.

Hellani,A., S.Coskun, A.Tbakhi, and S.Al Hassan. 2005. Clinical application of multiple displacement amplification in preimplantation genetic diagnosis. *Reprod. Biomed. Online.* 10: 376-380.

Hirasawa,R., H.Chiba, M.Kaneda, S.Tajima, E.Li, R.Jaenisch, and H.Sasaki. 2008. Maternal and zygotic Dnmt1 are necessary and sufficient for the maintenance of DNA methylation imprints during preimplantation development. *Genes Dev.* 22: 1607-1616.

Ho,T.H., B.Charlet, M.G.Poulos, G.Singh, M.S.Swanson, and T.A.Cooper. 2004. Muscleblind proteins regulate alternative splicing. *EMBO J.* 23: 3103-3112.

Hu,D.G., G.Webb, and N.Hussey. 2004. Aneuploidy detection in single cells using DNA array-based comparative genomic hybridization. *Mol. Hum. Reprod.* 10: 283-289.

Huggett,J., K.Dheda, S.Bustin, and A.Zumla. 2005. Real-time RT-PCR normalisation; strategies and considerations. *Genes Immun.* 6: 279-284.

Huntriss,J., M.Hinkins, B.Oliver, S.E.Harris, J.C.Beazley, A.J.Rutherford, R.G.Gosden, S.E.Lanzendorf, and H.M.Picton. 2004. Expression of mRNAs for DNA methyltransferases and methyl-CpG-binding proteins in the human female germ line, preimplantation embryos, and embryonic stem cells. *Mol. Reprod. Dev.* 67: 323-336.

Hurst,G.D., L.D.Hurst, and J.A.Barrett. 1995. Meiotic drive and myotonic dystrophy. *Nat. Genet.* 10: 132-133.

Ikeuchi,T., S.Igarashi, Y.Takiyama, O.Onodera, M.Oyake, H.Takano, R.Koide, H.Tanaka, and S.Tsuji. 1996. Non-Mendelian transmission in dentatorubral-pallidoluysian atrophy and Machado-Joseph disease: the mutant allele is preferentially transmitted in male meiosis. *Am. J. Hum. Genet.* 58: 730-733.

Imbert,G., C.Kretz, K.Johnson, and J.L.Mandel. 1993. Origin of the expansion mutation in myotonic dystrophy. *Nat. Genet.* 4: 72-76.

Inzunza,J., E.Iwarsson, M.Fridstrom, B.Rosenlund, P.Sjoblom, T.Hillensjo, E.Blennow, B.Jones, M.Nordenskjold, and L.Ahrlund-Richter. 1998. Application of single-needle blastomere biopsy in human preimplantation genetic diagnosis. *Prenat. Diagn.* 18: 1381-1388.

Jackson,K.V., E.S.Ginsburg, M.D.Hornstein, M.S.Rein, and R.N.Clarke. 1998. Multinucleation in normally fertilized embryos is associated with an accelerated ovulation induction response and lower implantation and pregnancy rates in in vitro fertilization-embryo transfer cycles. *Fertil. Steril.* 70: 60-66.

Jansen,G., P.J.Groenen, D.Bachner, P.H.Jap, M.Coerwinkel, F.Oerlemans, B.W.van den, B.Gohlsch, D.Pette, J.J.Plomp, P.C.Molenaar, M.G.Nederhoff, C.J.van Echteld, M.Dekker, A.Berns, H.Hameister, and B.Wieringa. 1996. Abnormal myotonic dystrophy protein kinase levels produce only mild myopathy in mice. *Nat. Genet.* 13: 316-324.

Jansen,G., P.Willems, M.Coerwinkel, W.Nillesen, H.Smeets, L.Vits, C.Howeler, H.Brunner, and B.Wieringa. 1994. Gonosomal mosaicism in myotonic dystrophy patients: involvement of mitotic events in (CTG)n repeat variation and selection against extreme expansion in sperm. *Am. J. Hum. Genet.* 54: 575-585.

Jeong,Y.J., H.W.Choi, H.S.Shin, X.S.Cui, N.H.Kim, G.L.Gerton, and J.H.Jun. 2005. Optimization of real time RT-PCR methods for the analysis of gene expression in mouse eggs and preimplantation embryos. *Mol. Reprod. Dev.* 71: 284-289.

Jones,G.M., B.Song, D.S.Cram, and A.O.Trounson. 2007. Optimization of a microarray based approach for deriving representative gene expression profiles from human oocytes. *Mol. Reprod. Dev.* 74: 8-17.

Jones,K.T. 2008. Meiosis in oocytes: predisposition to aneuploidy and its increased incidence with age. *Hum. Reprod. Update.* 14: 143-158.

Kakourou,G., S.Dhanjal, D.Daphnis, A.Doshi, S.Nuttall, S.Gotts, P.Serhal, J.Delhanty, J.Harper, and S.Sengupta. 2007. Preimplantation genetic diagnosis for myotonic dystrophy type 1: detection of crossover between the gene and the linked marker APOC2. *Prenat. Diagn.* 27: 111-116.

Kakourou,G., S.Dhanjal, T.Mamas, S.Gotts, A.Doshi, K.Fordham, P.Serhal, D.M.Ranieri, J.D.Delhanty, J.C.Harper, and S.B.SenGupta. 2008. Preimplantation genetic diagnosis for myotonic dystrophy type 1 in the UK. *Neuromuscul. Disord.* 18: 131-136.

Kanadia,R.N., K.A.Johnstone, A.Mankodi, C.Lungu, C.A.Thornton, D.Esson, A.M.Timmers, W.W.Hauswirth, and M.S.Swanson. 2003. A muscleblind knockout model for myotonic dystrophy. *Science* 302: 1978-1980.

Kattera,S. and C.Chen. 2003. Normal birth after microsurgical enucleation of tripronuclear human zygotes: case report. *Hum. Reprod.* 18: 1319-1322.

Kawamura,K., J.Kumagai, S.Sudo, S.Y.Chun, M.Pisarska, H.Morita, J.Toppari, P.Fu, J.D.Wade, R.A.Bathgate, and A.J.Hsueh. 2004. Paracrine regulation of mammalian oocyte maturation and male germ cell survival. *Proc. Natl. Acad. Sci. U. S. A* 101: 7323-7328.

Khajavi,M., A.M.Tari, N.B.Patel, K.Tsuji, D.R.Siwak, M.L.Meistrich, N.H.Terry, and T.Ashizawa. 2001. "Mitotic drive" of expanded CTG repeats in myotonic dystrophy type 1 (DM1). *Hum. Mol. Genet.* 10: 855-863.

Kim,S.A., J.A.Yoon, M.J.Kang, Y.M.Choi, S.J.Chae, and S.Y.Moon. 2008. An efficient and reliable DNA extraction method for preimplantation genetic diagnosis: a comparison of allele drop out and amplification rates using different single cell lysis methods. *Fertil. Steril.* [Epub-ahead of print]

Klesert,T.R., D.H.Cho, J.I.Clark, J.Maylie, J.Adelman, L.Snider, E.C.Yuen, P.Soriano, and S.J.Tapscott. 2000. Mice deficient in Six5 develop cataracts: implications for myotonic dystrophy. *Nat. Genet.* 25: 105-109.

Kocabas,A.M., J.Crosby, P.J.Ross, H.H.Otu, Z.Beyhan, H.Can, W.L.Tam, G.J.Rosa, R.G.Halgren, B.Lim, E.Fernandez, and J.B.Cibelli. 2006. The transcriptome of human oocytes. *Proc. Natl. Acad. Sci. U. S. A* 103: 14027-14032.

Kokkali,G., J.Traeger-Synodinos, C.Vrettou, D.Stavrou, G.M.Jones, D.S.Cram, E.Makrakis, A.O.Trounson, E.Kanavakis, and K.Pantos. 2007. Blastocyst biopsy versus cleavage stage biopsy and blastocyst transfer for preimplantation genetic diagnosis of beta-thalassaemia: a pilot study. *Hum. Reprod.* 22: 1443-1449.

Kremer,E.J., M.Pritchard, M.Lynch, S.Yu, K.Holman, E.Baker, S.T.Warren, D.Schlessinger, G.R.Sutherland, and R.I.Richards. 1991. Mapping of DNA instability at the fragile X to a trinucleotide repeat sequence p(CCG)n. *Science* 252: 1711-1714.

Kruse,B., D.Wohrle, P.Steinbach, and A.Gal. 2008. Does proximal myotonic myopathy show anticipation? *Hum. Mutat.* 29: E100-E102.

Kuliev,A. and Y.Verlinsky. 2005. Place of preimplantation diagnosis in genetic practice. *Am. J. Med. Genet. A* 134: 105-110.

Kuliev,A. and Y.Verlinsky. 2008. Preimplantation genetic diagnosis: technological advances to improve accuracy and range of applications. *Reprod. Biomed. Online.* 16: 532-538.

Kurihara,T. 2005. New classification and treatment for myotonic disorders. *Intern. Med.* 44: 1027-1032.

La Spada,A.R., E.M.Wilson, D.B.Lubahn, A.E.Harding, and K.H.Fischbeck. 1991. Androgen receptor gene mutations in X-linked spinal and bulbar muscular atrophy. *Nature* 352: 77-79.

Laberge,L., S.Veillette, J.Mathieu, J.Auclair, and M.Perron. 2007. The correlation of CTG repeat length with material and social deprivation in myotonic dystrophy. *Clin. Genet.* 71: 59-66.

Lahiri,D.K. and J.I.Nurnberger, Jr. 1991. A rapid non-enzymatic method for the preparation of HMW DNA from blood for RFLP studies. *Nucleic Acids Res.* 19: 5444.

Lalioti,M.D., H.S.Scott, C.Buresi, C.Rossier, A.Bottani, M.A.Morris, A.Malafosse, and S.E.Antonarakis. 1997. Dodecamer repeat expansion in cystatin B gene in progressive myoclonus epilepsy. *Nature* 386: 847-851.

Larsen,W.J., S.E.Wert, and G.D.Brunner. 1986. A dramatic loss of cumulus cell gap junctions is correlated with germinal vesicle breakdown in rat oocytes. *Dev. Biol.* 113: 517-521.

Laurent,L.C., J.Chen, I.Ulitsky, F.J.Mueller, C.Lu, R.Shamir, J.B.Fan, and J.F.Loring. 2008. Comprehensive microRNA profiling reveals a unique human embryonic stem cell signature dominated by a single seed sequence. *Stem Cells* 26: 1506-1516.

Lavedan,C., H.Hofmann-Radvanyi, P.Shelbourne, J.P.Rabes, C.Duros, D.Savoy, I.Dehaupas, S.Luce, K.Johnson, and C.Junien. 1993. Myotonic dystrophy: size- and sex-dependent dynamics of CTG meiotic instability, and somatic mosaicism. *Am. J. Hum. Genet.* 52: 875-883.

Lavery,S.A., R.Aurell, C.Turner, C.Castello, A.Veiga, P.N.Barri, and R.M.Winston. 2002. Preimplantation genetic diagnosis: patients' experiences and attitudes. *Hum. Reprod.* 17: 2464-2467.

Le Ber, I, M.Martinez, D.Campion, A.Laquerriere, C.Betard, G.Bassez, C.Girard, P.Saugier-Veber, G.Raux, N.Sergeant, P.Magnier, T.Maisonobe, B.Eymard, C.Duyckaerts, A.Delacourte, T.Frebourg, and D.Hannequin. 2004. A non-DM1, non-DM2 multisystem myotonic disorder with frontotemporal dementia: phenotype and suggestive mapping of the DM3 locus to chromosome 15q21-24. *Brain* 127: 1979-1992.

Le Caignec.C., C.Spits, K.Sermon, R.M.De, B.Thienpont, S.Debrock, C.Staessen, Y.Moreau, J.P.Fryns, S.A.Van, I.Liebaers, and J.R.Vermeesch. 2006. Single-cell chromosomal imbalances detection by array CGH. *Nucleic Acids Res.* 34: e68.

Leeflang,E.P., M.S.McPeek, and N.Arnheim. 1996. Analysis of meiotic segregation, using single-sperm typing: meiotic drive at the myotonic dystrophy locus. *Am. J. Hum. Genet.* 59: 896-904.

Lewis,B.P., C.B.Burge, and D.P.Bartel. 2005. Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. *Cell* 120: 15-20.

Lewis,C.M., T.Pinel, J.C.Whittaker, and A.H.Handyside. 2001. Controlling misdiagnosis errors in preimplantation genetic diagnosis: a comprehensive model encompassing extrinsic and intrinsic sources of error. *Hum. Reprod.* 16: 43-50.

Li,Q., L.J.McKenzie, and M.M.Matzuk. 2008. Revisiting oocyte-somatic cell interactions: In search of novel intrafollicular predictors and regulators of oocyte developmental competence. *Mol. Hum. Reprod.*14 (12): 673-8

Li,S.S., Y.H.Liu, C.N.Tseng, and S.Singh. 2006. Analysis of gene expression in single human oocytes and preimplantation embryos. *Biochem. Biophys. Res. Commun.* 340: 48-53.

Liquori,C.L., K.Ricker, M.L.Moseley, J.F.Jacobsen, W.Kress, S.L.Naylor, J.W.Day, and L.P.Ranum. 2001. Myotonic dystrophy type 2 caused by a CCTG expansion in intron 1 of ZNF9. *Science* 293: 864-867.

Liss,B. 2002. Improved quantitative real-time RT-PCR for expression profiling of individual cells. *Nucleic Acids Res.* 30: e89.

Liu,J., A.E.Van den, and A.Van Steirteghem. 1993. The in-vitro and in-vivo developmental potential of frozen and non-frozen biopsied 8-cell mouse embryos. *Hum. Reprod.* 8: 1481-1486.

Magee,A.C. and A.E.Hughes. 1998. Segregation distortion in myotonic dystrophy. *J. Med. Genet.* 35: 1045-1046.

Magee,A.C., A.E.Hughes, A.Kidd, M.A.Lopez De, A.M.Cobo, K.Kelly, J.Dean, and N.C.Nevin. 2002. Reproductive counselling for women with myotonic dystrophy. *J. Med. Genet.* 39: E15.

Magli,M.C., L.Gianaroli, N.Grieco, E.Cefalu, G.Ruvolo, and A.P.Ferraretti. 2006. Cryopreservation of biopsied embryos at the blastocyst stage. *Hum. Reprod.* 21: 2656-2660.

Mahadevan,M., C.Tsilfidis, L.Sabourin, G.Shutler, C.Amemiya, G.Jansen, C.Neville, M.Narang, J.Barcelo, K.O'Hoy, *et al*. 1992. Myotonic dystrophy mutation: an unstable CTG repeat in the 3' untranslated region of the gene. *Science* 255: 1253-1255.

Mahadevan,M.S., C.Amemiya, G.Jansen, L.Sabourin, S.Baird, C.E.Neville, N.Wormskamp, B.Segers, M.Batzer, J.Lamerdin, *et al*. 1993a. Structure and genomic sequence of the myotonic dystrophy (DM kinase) gene. *Hum. Mol. Genet.* 2: 299-304.

Mahadevan,M.S., M.A.Foitzik, L.C.Surh, and R.G.Korneluk. 1993b. Characterization and polymerase chain reaction (PCR) detection of an Alu deletion polymorphism in total linkage disequilibrium with myotonic dystrophy. *Genomics* 15: 446-448.

Mak,W., T.B.Nesterova, N.M.de, R.Appanah, S.Yamanaka, A.P.Otte, and N.Brockdorff. 2004. Reactivation of the paternal X chromosome in early mouse embryos. *Science* 303: 666-669.

Mamo,S., A.B.Gal, S.Bodo, and A.Dinnyes. 2007. Quantitative evaluation and selection of reference genes in mouse oocytes and embryos cultured in vivo and in vitro. *BMC. Dev. Biol.* 7: 14.

Mamo,S., A.B.Gal, Z.Polgar, and A.Dinnyes. 2008. Expression profiles of the pluripotency marker gene POU5F1 and validation of reference genes in rabbit oocytes and preimplantation stage embryos. *BMC. Mol. Biol.* 9: 67.

Mankodi,A., E.Logigian, L.Callahan, C.McClain, R.White, D.Henderson, M.Krym, and C.A.Thornton. 2000. Myotonic dystrophy in transgenic mice expressing an expanded CUG repeat. *Science* 289: 1769-1773.

Mankodi,A., M.P.Takahashi, H.Jiang, C.L.Beck, W.J.Bowers, R.T.Moxley, S.C.Cannon, and C.A.Thornton. 2002. Expanded CUG repeats trigger aberrant splicing of ClC-1 chloride channel pre-mRNA and hyperexcitability of skeletal muscle in myotonic dystrophy. *Mol. Cell* 10: 35-44.

Manor,D., S.Kol, N.Lewit, A.Lightman, D.Stein, M.Pillar, and J.Itskovitz-Eldor. 1996. Undocumented embryos: do not trash them, FISH them. *Hum. Reprod.* 11: 2502-2506.

Mantzouratou,A., A.Mania, E.Fragouli, L.Xanthopoulou, S.Tashkandi, K.Fordham, D.M.Ranieri, A.Doshi, S.Nuttall, J.C.Harper, P.Serhal, and J.D.Delhanty. 2007. Variable aneuploidy mechanisms in embryos from couples with poor reproductive histories undergoing preimplantation genetic screening. *Hum. Reprod.* 22: 1844-1853.

Marchini,C., R.Lonigro, L.Verriello, L.Pellizzari, P.Bergonzi, and G.Damante. 2000. Correlations between individual clinical manifestations and CTG repeat amplification in myotonic dystrophy. *Clin. Genet.* 57: 74-82.

Martorell,L., A.M.Cobo, M.Baiget, M.Naudo, J.J.Poza, and J.Parra. 2007. Prenatal diagnosis in myotonic dystrophy type 1. Thirteen years of experience: implications for reproductive counselling in DM1 families. *Prenat. Diagn.* 27: 68-72.

Martorell,L., K.Johnson, C.A.Boucher, and M.Baiget. 1997. Somatic instability of the myotonic dystrophy (CTG)n repeat during human fetal development. *Hum. Mol. Genet.* 6: 877-880.

Martorell,L., J.M.Martinez, N.Carey, K.Johnson, and M.Baiget. 1995. Comparison of CTG repeat length expansion and clinical progression of myotonic dystrophy over a five year period. *J. Med. Genet.* 32: 593-596.

Martorell,L., D.G.Monckton, J.Gamez, and M.Baiget. 2000. Complex patterns of male germline instability and somatic mosaicism in myotonic dystrophy type 1. *Eur. J. Hum. Genet.* 8: 423-430.

Martorell,L., D.G.Monckton, J.Gamez, K.J.Johnson, I.Gich, d.M.Lopez, and M.Baiget. 1998. Progression of somatic CTG repeat length heterogeneity in the blood cells of myotonic dystrophy patients. *Hum. Mol. Genet.* 7: 307-312.

Martorell,L., D.G.Monckton, A.Sanchez, M.A.Lopez De, and M.Baiget. 2001. Frequency and stability of the myotonic dystrophy type 1 premutation. *Neurology* 56: 328-335.

Massari,A., G.Novelli, A.Colosimo, F.Sangiuolo, G.Palka, G.Calabrese, L.Camurri, G.Ghirardini, G.Milani, C.Giorlandino, G.Gazzanelli, M.Malatesta, C.Romanini, and B.Dallapiccola. 1996. Non-invasive early prenatal molecular diagnosis using retrieved transcervical trophoblast cells. *Hum. Genet.* 97: 150-155.

Mateizel,I., T.N.De, U.Ullmann, G.Cauffman, K.Sermon, d.Van, V, R.M.De, E.Degreef, P.Devroey, I.Liebaers, and S.A.Van. 2006. Derivation of human embryonic stem cell lines from embryos obtained after IVF and after PGD for monogenic disorders. *Hum. Reprod.* 21: 503-511.

Matsuura,T., T.Yamagata, D.L.Burgess, A.Rasmussen, R.P.Grewal, K.Watase, M.Khajavi, A.E.McCall, C.F.Davis, L.Zu, M.Achari, S.M.Pulst, E.Alonso, J.L.Noebels, D.L.Nelson, H.Y.Zoghbi, and T.Ashizawa. 2000. Large expansion of the ATTCT pentanucleotide repeat in spinocerebellar ataxia type 10. *Nat. Genet.* 26: 191-194.

McArthur,S.J., D.Leigh, J.T.Marshall, K.A.de Boer, and R.P.Jansen. 2005. Pregnancies and live births after trophectoderm biopsy and preimplantation genetic testing of human blastocysts. *Fertil. Steril.* 84: 1628-1636.

McArthur,S.J., D.Leigh, J.T.Marshall, A.J.Gee, K.A.de Boer, and R.P.Jansen. 2008. Blastocyst trophectoderm biopsy and preimplantation genetic diagnosis for familial monogenic disorders and chromosomal translocations. *Prenat. Diagn.* 28: 434-442.

McKenzie,L.J., S.A.Pangas, S.A.Carson, E.Kovanci, P.Cisneros, J.E.Buster, P.Amato, and M.M.Matzuk. 2004. Human cumulus granulosa cell gene expression: a predictor of fertilization and embryo selection in women undergoing IVF. *Hum. Reprod.* 19: 2869-2874.

Mehlmann,L.M. 2005. Stops and starts in mammalian oocytes: recent advances in understanding the regulation of meiotic arrest and oocyte maturation. *Reproduction.* 130: 791-799.

Menezo,Y., Jr., G.Russo, E.Tosti, M.S.El, and M.Benkhalifa. 2007. Expression profile of genes coding for DNA repair in human oocytes using pangenomic microarrays, with a special focus on ROS linked decays. *J. Assist. Reprod. Genet.* 24: 513-520.

Meola,G. 2000. Clinical and genetic heterogeneity in myotonic dystrophies. *Muscle Nerve* 23: 1789-1799.

Mi,H., N.Guo, A.Kejariwal, and P.D.Thomas. 2007. PANTHER version 6: protein sequence and function evolution data with expanded representation of biological pathways. *Nucleic Acids Res.* 35: D247-D252.

Mi,H., B.Lazareva-Ulitsky, R.Loo, A.Kejariwal, J.Vandergriff, S.Rabkin, N.Guo, A.Muruganujan, O.Doremieux, M.J.Campbell, H.Kitano, and P.D.Thomas. 2005. The PANTHER database of protein families, subfamilies, functions and pathways. *Nucleic Acids Res.* 33: D284-D288.

Mirkin,S.M. 2006. DNA structures, repeat expansions and human hereditary disorders. *Curr. Opin. Struct. Biol.* 16: 351-358.

Monckton,D.G., L.J.Wong, T.Ashizawa, and C.T.Caskey. 1995. Somatic mosaicism, germline expansions, germline reversions and intergenerational reductions in myotonic dystrophy males: small pool PCR analyses. *Hum. Mol. Genet.* 4: 1-8.

Monk,M., R.L.Adams, and A.Rinaldi. 1991. Decrease in DNA methylase activity during preimplantation development in the mouse. *Development* 112: 189-192.

Moraes,K.C., C.J.Wilusz, and J.Wilusz. 2006. CUG-BP binds to RNA substrates and recruits PARN deadenylase. *RNA.* 12: 1084-1091.

Moutou,C., N.Gardes, J.C.Nicod, and S.Viville. 2007. Strategies and outcomes of PGD of familial adenomatous polyposis. *Mol. Hum. Reprod.* 13: 95-101.

Moutou,C., N.Gardes, and S.Viville. 2003. Duplex PCR for preimplantation genetic diagnosis (PGD) of spinal muscular atrophy. *Prenat. Diagn.* 23: 685-689.

Mtango,N.R., S.Potireddy, and K.E.Latham. 2008. Expression of MicroRNA Processing Machinery Genes in Rhesus Monkey Oocytes and Embryos of Different Developmental Potentials. *Mol. Reprod. Dev.*76: 255-269.

Murchison,E.P., P.Stein, Z.Xuan, H.Pan, M.Q.Zhang, R.M.Schultz, and G.J.Hannon. 2007. Critical roles for Dicer in the female germline. *Genes Dev.* 21: 682-693.

Nag,D.K. 2003. Trinucleotide repeat expansions: timing is everything. *Trends Mol. Med.* 9: 455-457.

Nakagawa,M., H.Yamada, I.Higuchi, Y.Kaminishi, T.Miki, K.Johnson, and M.Osame. 1994. A case of paternally inherited congenital myotonic dystrophy. *J. Med. Genet.* 31: 397-400.

Nakamori,M., T.Kimura, H.Fujimura, M.P.Takahashi, and S.Sakoda. 2007. Altered mRNA splicing of dystrophin in type 1 myotonic dystrophy. *Muscle Nerve* 36: 251-257.

Navidi,W. and N.Arnheim. 1991. Using PCR in preimplantation genetic disease diagnosis. *Hum. Reprod.* 6: 836-849.

Neilson,L., A.Andalibi, D.Kang, C.Coutifaris, J.F.Strauss, III, J.A.Stanton, and D.P.Green. 2000. Molecular phenotype of the human oocyte by PCR-SAGE. *Genomics* 63: 13-24.

Nekkebroeck,J., M.Bonduelle, S.Desmyttere, B.W.Van den, and I.Ponjaert-Kristoffersen. 2008. Socio-emotional and language development of 2-year-old children born after PGD/PGS, and parental well-being. *Hum. Reprod.* 23: 1849-1857.

Neville,C.E., M.S.Mahadevan, J.M.Barcelo, and R.G.Korneluk. 1994. High resolution genetic analysis suggests one ancestral predisposing haplotype for the origin of the myotonic dystrophy mutation. *Hum. Mol. Genet.* 3: 45-51.

Nichol,K. and C.E.Pearson. 2002. CpG methylation modifies the genetic stability of cloned repeat sequences. *Genome Res.* 12: 1246-1256.

Nolin,S.L., W.T.Brown, A.Glicksman, G.E.Houck, Jr., A.D.Gargano, A.Sullivan, V.Biancalana, K.Brondum-Nielsen, H.Hjalgrim, E.Holinski-Feder, F.Kooy, J.Longshore, J.Macpherson, J.L.Mandel, G.Matthijs, F.Rousseau, P.Steinbach, M.L.Vaisanen, K.H.von, and S.L.Sherman. 2003. Expansion of the fragile X CGG repeat in females with premutation or intermediate alleles. *Am. J. Hum. Genet.* 72: 454-464.

Norppa,H. and G.C.Falck. 2003. What do human micronuclei contain? *Mutagenesis* 18: 221-233.

Nothias,J.Y., S.Majumder, K.J.Kaneko, and M.L.DePamphilis. 1995. Regulation of gene expression at the beginning of mammalian development. *J. Biol. Chem.* 270: 22077-22080.

O'Cochlain,D.F., C.Perez-Terzic, S.Reyes, G.C.Kane, A.Behfar, D.M.Hodgson, J.A.Strommen, X.K.Liu, B.W.van den, D.G.Wansink, B.Wieringa, and A.Terzic. 2004. Transgenic overexpression of human DMPK accumulates into hypertrophic cardiomyopathy, myotonic myopathy and hypotension traits of myotonic dystrophy. *Hum. Mol. Genet.* 13: 2505-2518.

Osborne,R.J. and C.A.Thornton. 2006. RNA-dominant diseases. *Hum. Mol. Genet.* 15 Spec No 2: R162-R169.

Pan,H., H.M.Lin, W.Y.Ku, T.C.Li, S.Y.Li, C.C.Lin, and K.M.Hsiao. 2001. Haplotype analysis of the myotonic dystrophy type 1 (DM1) locus in Taiwan: implications for low prevalence and founder mutations of Taiwanese myotonic dystrophy type 1. *Eur. J. Hum. Genet.* 9: 638-641.

Paszkiewicz,G.M., E.A.Timm, Jr., M.C.Mahoney, P.K.Wallace, M.A.Sullivan Nasca, T.L.Tammela, A.Hutson, and J.L.Pauly. 2008. Increased human buccal cell autofluorescence is a candidate biomarker of tobacco smoking. *Cancer Epidemiol. Biomarkers Prev.* 17: 239-244.

Patrizio,P., E.Fragouli, V.Bianchi, A.Borini, and D.Wells. 2007. Molecular methods for selection of the ideal oocyte. *Reprod. Biomed. Online.* 15: 346-353.

Pavlidis,P., Q.Li, and W.S.Noble. 2003. The effect of replication on gene expression microarray experiments. *Bioinformatics.* 19: 1620-1627.

Peng,W., H.Takabayashi, and K.Ikawa. 2007. Whole genome amplification from single cells in preimplantation genetic diagnosis and prenatal diagnosis. *Eur. J. Obstet. Gynecol. Reprod. Biol.* 131: 13-20.

Pergament,E. 1991. Preimplantation diagnosis: a patient perspective. *Prenat. Diagn.* 11: 493-500.

Pickering,S., N.Polidoropoulos, J.Caller, P.Scriven, C.M.Ogilvie, and P.Braude. 2003. Strategies and outcomes of the first 100 cycles of preimplantation genetic diagnosis at the Guy's and St. Thomas' Center. *Fertil. Steril.* 79: 81-90.

Picton,H.M., S.E.Harris, W.Muruvi, and E.L.Chambers. 2008. The in vitro growth and maturation of follicles. *Reproduction.* 136: 703-715.

Pierce,K.E., J.E.Rice, J.A.Sanchez, and L.J.Wangh. 2003. Detection of cystic fibrosis alleles from single cells using molecular beacons and a novel method of asymmetric real-time PCR. *Mol. Hum. Reprod.* 9: 815-820.

Pinborg,A., A.Loft, L.Schmidt, and A.N.Andersen. 2003. Morbidity in a Danish national cohort of 472 IVF/ICSI twins, 1132 non-IVF/ICSI twins and 634 IVF/ICSI singletons: health-related and social implications for the children and their families. *Hum. Reprod.* 18: 1234-1243.

Piyamongkol,W., M.G.Bermudez, J.C.Harper, and D.Wells. 2003. Detailed investigation of factors influencing amplification efficiency and allele drop-out in single cell PCR: implications for preimplantation genetic diagnosis. *Mol. Hum. Reprod.* 9: 411-420.

Piyamongkol,W., J.C.Harper, J.D.Delhanty, and D.Wells. 2001a. PGD protocols using multiplex fluorescent PCR. *Reprod. Biomed. Online.* 2: 212-214.

Piyamongkol,W., J.C.Harper, J.K.Sherlock, A.Doshi, P.F.Serhal, J.D.Delhanty, and D.Wells. 2001b. A successful strategy for preimplantation genetic diagnosis of myotonic dystrophy using multiplex fluorescent PCR. *Prenat. Diagn.* 21: 223-232.

Quackenbush,J. 2001. Computational analysis of microarray data. *Nat. Rev. Genet.* 2: 418-427.

Ranum,L.P. and T.A.Cooper. 2006. RNA-mediated neuromuscular disorders. *Annu. Rev. Neurosci.* 29: 259-277.

Ranum,L.P. and J.W.Day. 2004. Pathogenic RNA repeats: an expanding role in genetic disease. *Trends Genet.* 20: 506-512.

Ranum,L.P., P.F.Rasmussen, K.A.Benzow, M.D.Koob, and J.W.Day. 1998. Genetic mapping of a second myotonic dystrophy locus. *Nat. Genet.* 19: 196-198.

Ray,P.F. and A.H.Handyside. 1996. Increasing the denaturation temperature during the first cycles of amplification reduces allele dropout from single cells for preimplantation genetic diagnosis. *Mol. Hum. Reprod.* 2: 213-218.

Rechitsky,S., A.Kuliev, I.Tur-Kaspa, R.Morris, and Y.Verlinsky. 2004. Preimplantation genetic diagnosis with HLA matching. *Reprod. Biomed. Online.* 9: 210-221.

Rechitsky,S., C.Strom, O.Verlinsky, T.Amet, V.Ivakhnenko, V.Kukharenko, A.Kuliev, and Y.Verlinsky. 1998. Allele dropout in polar bodies and blastomeres. *J. Assist. Reprod. Genet.* 15: 253-257.

Reik,W., W.Dean, and J.Walter. 2001. Epigenetic reprogramming in mammalian development. *Science* 293: 1089-1093.

Renwick,P.J., C.M.Lewis, S.Abbs, and C.M.Ogilvie. 2007. Determination of the genetic status of cleavage-stage human embryos by microsatellite marker analysis following multiple displacement amplification. *Prenat. Diagn.* 27: 206-215.

Renwick,P.J., J.Trussler, E.Ostad-Saffari, H.Fassihi, C.Black, P.Braude, C.M.Ogilvie, and S.Abbs. 2006. Proof of principle and first cases using preimplantation genetic haplotyping--a paradigm shift for embryo diagnosis. *Reprod. Biomed. Online.* 13: 110-119.

Rice,J.E., J.A.Sanchez, K.E.Pierce, and L.J.Wangh. 2002. Real-time PCR with molecular beacons provides a highly accurate assay for detection of Tay-Sachs alleles in single cells. *Prenat. Diagn.* 22: 1130-1134.

Richard,F.J. 2007. Regulation of meiotic maturation. *J. Anim Sci.* 85: E4-E6.

Ricker,K., T.Grimm, M.C.Koch, C.Schneider, W.Kress, C.D.Reimers, W.Schulte-Mattler, B.Mueller-Myhsok, K.V.Toyka, and C.R.Mueller. 1999. Linkage of proximal myotonic myopathy to chromosome 3q. *Neurology* 52: 170-171.

Riess,O., J.T.Epplen, G.Amoiridis, H.Przuntek, and L.Schols. 1997. Transmission distortion of the mutant alleles in spinocerebellar ataxia. *Hum. Genet.* 99: 282-284.

Robert,C., S.McGraw, L.Massicotte, M.Pravetoni, F.Gandolfi, and M.A.Sirard. 2002. Quantification of housekeeping transcript levels during the development of bovine preimplantation embryos. *Biol. Reprod.* 67: 1465-1472.

Rolland,Y., B.Mazieres, and M.Laroche. 1999. [Steinert disease associated with Klinefelter's syndrome]. *Rev. Neurol. (Paris)* 155: 229-230.

Sahu,B., O.Ozturk, N.Deo, K.Fordham, M.Ranierri, and P.Serhal. 2008. Response to controlled ovarian stimulation and oocyte quality in women with myotonic dystrophy type I. *J. Assist. Reprod. Genet.* 25: 1-5.

Salehi,L.B., E.Bonifazi, E.D.Stasio, M.Gennarelli, A.Botta, L.Vallo, R.Iraci, R.Massa, G.Antonini, C.Angelini, and G.Novelli. 2007. Risk prediction for clinical phenotype in myotonic dystrophy type 1: data from 2,650 patients. *Genet. Test.* 11: 84-90.

Salpekar,A., J.Huntriss, V.Bolton, and M.Monk. 2001. The use of amplified cDNA to investigate the expression of seven imprinted genes in human oocytes and preimplantation embryos. *Mol. Hum. Reprod.* 7: 839-844.

Sarkar,P.S., B.Appukuttan, J.Han, Y.Ito, C.Ai, W.Tsai, Y.Chai, J.T.Stout, and S.Reddy. 2000. Heterozygous loss of Six5 in mice is sufficient to cause ocular cataracts. *Nat. Genet.* 25: 110-114.

Sarkar,P.S., S.Paul, J.Han, and S.Reddy. 2004. Six5 is required for spermatogenic cell survival and spermiogenesis. *Hum. Mol. Genet.* 13: 1421-1431.

Savouret,C., E.Brisson, J.Essers, R.Kanaar, A.Pastink, R.H.te, C.Junien, and G.Gourdon. 2003. CTG repeat instability and size variation timing in DNA repair-deficient mice. *EMBO J.* 22: 2264-2273.

Savouret,C., C.Garcia-Cordier, J.Megret, R.H.te, C.Junien, and G.Gourdon. 2004. MSH2-dependent germinal CTG repeat expansions are produced continuously in spermatogonia from DM1 transgenic mice. *Mol. Cell Biol.* 24: 629-637.

Schara,U. and B.G.Schoser. 2006. Myotonic dystrophies type 1 and 2: a summary on current aspects. *Semin. Pediatr. Neurol.* 13: 71-79.

Schmittgen,T.D. 2008. Regulation of microRNA processing in development, differentiation and cancer. *J. Cell Mol. Med.*12: 1811-1819.

Schroeder,A., O.Mueller, S.Stocker, R.Salowsky, M.Leiber, M.Gassmann, S.Lightfoot, W.Menzel, M.Granzow, and T.Ragg. 2006. The RIN: an RNA integrity number for assigning integrity values to RNA measurements. *BMC. Mol. Biol.* 7: 3.

Sermon,K., V.A.De, d.Van, V, S.Seneca, W.Lissens, H.Joris, M.Vandervorst, S.A.Van, and I.Liebaers. 1998a. Fluorescent PCR and automated fragment analysis for the clinical application of preimplantation genetic diagnosis of myotonic dystrophy (Steinert's disease). *Mol. Hum. Reprod.* 4: 791-796.

Sermon,K., V.Goossens, S.Seneca, W.Lissens, A.De Vos, M.Vandervorst, A.Van Steirteghem, and I.Liebaers. 1998b. Preimplantation diagnosis for Huntington's disease (HD): clinical application and analysis of the HD expansion in affected embryos. *Prenat. Diagn.* 18: 1427-1436.

Sermon,K., W.Lissens, H.Joris, S.Seneca, S.Desmyttere, P.Devroey, A.Van Steirteghem, and I.Liebaers. 1997. Clinical application of preimplantation diagnosis for myotonic dystrophy. *Prenat. Diagn.* 17: 925-932.

Sermon,K., S.Seneca, M.De Rycke, V.Goossens, d.Van, V, A.De Vos, P.Platteau, W.Lissens, A.Van Steirteghem, and I.Liebaers. 2001. PGD in the lab for triplet repeat diseases - myotonic dystrophy, Huntington's disease and Fragile-X syndrome. *Mol. Cell Endocrinol.* 183 Suppl 1: S77-S85.

Sermon,K., A.Van Steirteghem, and I.Liebaers. 2004. Preimplantation genetic diagnosis. *Lancet* 363: 1633-1641.

Seznec,H., O.Agbulut, N.Sergeant, C.Savouret, A.Ghestem, N.Tabti, J.C.Willer, L.Ourth, C.Duros, E.Brisson, C.Fouquet, G.Butler-Browne, A.Delacourte, C.Junien, and G.Gourdon. 2001. Mice transgenic for the human myotonic dystrophy region with expanded CTG repeats display muscular and brain abnormalities. *Hum. Mol. Genet.* 10: 2717-2726.

Sher,G., L.Keskintepe, M.Keskintepe, G.Maassarani, D.Tortoriello, and S.Brody. 2009. Genetic analysis of human embryos by metaphase comparative genomic hybridization (mCGH) improves efficiency of IVF by increasing embryo implantation rate and reducing multiple pregnancies and spontaneous miscarriages. *Fertil. Steril.*[Epub ahead of print]

Sherlock,J., V.Cirigliano, M.Petrou, B.Tutschek, and M.Adinolfi. 1998. Assessment of diagnostic quantitative fluorescent multiplex polymerase chain reaction assays performed on single cells. *Ann. Hum. Genet.* 62 (Pt 1): 9-23.

Sobczak,K., M.de Mezer, G.Michlewski, J.Krol, and W.J.Krzyzosiak. 2003. RNA structure of trinucleotide repeats associated with human neurological diseases. *Nucleic Acids Res.* 31: 5469-5482.

Soini,S. 2007. Preimplantation genetic diagnosis (PGD) in Europe: diversity of legislation a challenge to the community and its citizens. *Med. Law* 26: 309-323.

Soini,S., D.Ibarreta, V.Anastasiadou, S.Ayme, S.Braga, M.Cornel, D.A.Coviello, G.Evers-Kiebooms, J.Geraedts, L.Gianaroli, J.Harper, G.Kosztolanyi, K.Lundin, E.Rodrigues-Cerezo, K.Sermon, J.Sequeiros, L.Tranebjaerg, and H.Kaariainen. 2006. The interface between assisted reproductive technologies and genetics: technical, social, ethical and legal issues. *Eur. J. Hum. Genet.* 14: 588-645.

Sousa,M., A.Barros, J.Silva, and J.Tesarik. 1997. Developmental changes in calcium content of ultrastructurally distinct subcellular compartments of preimplantation human embryos. *Mol. Hum. Reprod.* 3: 83-90.

Spanos,S., S.Rice, P.Karagiannis, D.Taylor, D.L.Becker, R.M.Winston, and K.Hardy. 2002. Caspase activity and expression of cell death genes during development of human preimplantation embryos. *Reproduction.* 124: 353-363.

Spits,C., M.De Rycke, W.Verpoest, W.Lissens, A.Van Steirteghem, I.Liebaers, and K.Sermon. 2006. Preimplantation genetic diagnosis for Marfan syndrome. *Fertil. Steril.* 86: 310-320.

Spits,C. and K.Sermon. 2009. PGD for monogenic disorders: aspects of molecular biology. *Prenat. Diagn.* 29: 50-56.

Staessen,C. and A.C.Van Steirteghem. 1997. The chromosomal constitution of embryos developing from abnormally fertilized oocytes after intracytoplasmic sperm injection and conventional in-vitro fertilization. *Hum. Reprod.* 12: 321-327.

Stanton,J.A., A.B.Macgregor, C.Mason, M.Dameh, and D.P.Green. 2007. Building comparative gene expression databases for the mouse preimplantation embryo using a pipeline approach to UniGene. *Mol. Hum. Reprod.* 13: 713-720.

Steinbach,P., D.Glaser, W.Vogel, M.Wolf, and S.Schwemmle. 1998. The DMPK gene of severely affected myotonic dystrophy patients is hypermethylated proximal to the largely expanded CTG repeat. *Am. J. Hum. Genet.* 62: 278-285.

Steuerwald,N., J.Cohen, R.J.Herrera, and C.A.Brenner. 2000. Quantification of mRNA in single oocytes and embryos by real-time rapid cycle fluorescence monitored RT-PCR. *Mol. Hum. Reprod.* 6: 448-453.

Sturmey,R.G., P.J.O'Toole, and H.J.Leese. 2006. Fluorescence resonance energy transfer analysis of mitochondrial:lipid association in the porcine oocyte. *Reproduction.* 132: 829-837.

Su,Y.Q., K.Sugiura, K.Wigglesworth, M.J.O'Brien, J.P.Affourtit, S.A.Pangas, M.M.Matzuk, and J.J.Eppig. 2008. Oocyte regulation of metabolic cooperativity between mouse cumulus cells and oocytes: BMP15 and GDF9 control cholesterol biosynthesis in cumulus cells. *Development* 135: 111-121.

Suetake,I., F.Shinozaki, J.Miyagawa, H.Takeshima, and S.Tajima. 2004. DNMT3L stimulates the DNA methylation activity of Dnmt3a and Dnmt3b through a direct interaction. *J. Biol. Chem.* 279: 27816-27823.

Sugiura,K., Y.Q.Su, F.J.Diaz, S.A.Pangas, S.Sharma, K.Wigglesworth, M.J.O'Brien, M.M.Matzuk, S.Shimasaki, and J.J.Eppig. 2007. Oocyte-derived BMP15 and FGFs cooperate to promote glycolysis in cumulus cells. *Development* 134: 2593-2603.

Sultan,K.M., S.Munne, G.D.Palermo, M.Alikani, and J.Cohen. 1995. Chromosomal status of uni-pronuclear human zygotes following in-vitro fertilization and intracytoplasmic sperm injection. *Hum. Reprod.* 10: 132-136.

Sun,Q.Y., G.M.Wu, L.Lai, K.W.Park, R.Cabot, H.T.Cheong, B.N.Day, R.S.Prather, and H.Schatten. 2001. Translocation of active mitochondria during pig oocyte maturation, fertilization and early embryo development in vitro. *Reproduction.* 122: 155-163.

Swanson,A., E.Strawn, E.Lau, and D.Bick. 2007. Preimplantation genetic diagnosis: technology and clinical applications. *WMJ.* 106: 145-151.

Synnergren,J., T.L.Giesler, S.Adak, R.Tandon, K.Noaksson, A.Lindahl, P.Nilsson, D.Nelson, B.Olsson, M.C.Englund, S.Abbot, and P.Sartipy. 2007. Differentiating human embryonic stem cells express a unique housekeeping gene signature. *Stem Cells* 25: 473-480.

Tanaka,Y., Y.Suzuki, N.Shimozawa, E.Nanba, and N.Kondo. 2000. Congenital myotonic dystrophy: report of paternal transmission. *Brain Dev.* 22: 132-134.

Tang,F., M.Kaneda, D.O'Carroll, P.Hajkova, S.C.Barton, Y.A.Sun, C.Lee, A.Tarakhovsky, K.Lao, and M.A.Surani. 2007. Maternal microRNAs are essential for mouse zygotic development. *Genes Dev.* 21: 644-648.

Tarin,J.J., J.Conaghan, R.M.Winston, and A.H.Handyside. 1992. Human embryo biopsy on the 2nd day after insemination for preimplantation diagnosis: removal of a quarter of embryo retards cleavage. *Fertil. Steril.* 58: 970-976.

Tesarik,J., V.Kopecny, M.Plachot, and J.Mandelbaum. 1987. Ultrastructural and autoradiographic observations on multinucleated blastomeres of human cleaving embryos obtained by in-vitro fertilization. *Hum. Reprod.* 2: 127-136.

Thomas,P.D., M.J.Campbell, A.Kejariwal, H.Mi, B.Karlak, R.Daverman, K.Diemer, A.Muruganujan, and A.Narechania. 2003. PANTHER: a library of protein families and subfamilies indexed by function. *Genome Res.* 13: 2129-2141.

Thomson,J.A., J.Itskovitz-Eldor, S.S.Shapiro, M.A.Waknitz, J.J.Swiergiel, V.S.Marshall, and J.M.Jones. 1998. Embryonic stem cell lines derived from human blastocysts. *Science* 282: 1145-1147.

Thornhill,A.R., C.E.Die-Smulders, J.P.Geraedts, J.C.Harper, G.L.Harton, S.A.Lavery, C.Moutou, M.D.Robinson, A.G.Schmutzler, P.N.Scriven, K.D.Sermon, and L.Wilton. 2005. ESHRE PGD Consortium 'Best practice guidelines for clinical preimplantation genetic diagnosis (PGD) and preimplantation genetic screening (PGS)'. *Hum. Reprod.* 20: 35-48.

Thornhill,A.R., J.A.McGrath, R.A.Eady, P.R.Braude, and A.H.Handyside. 2001. A comparison of different lysis buffers to assess allele dropout from single cells for preimplantation genetic diagnosis. *Prenat. Diagn.* 21: 490-497.

Timchenko,N.A., Z.J.Cai, A.L.Welm, S.Reddy, T.Ashizawa, and L.T.Timchenko. 2001. RNA CUG repeats sequester CUGBP1 and alter protein levels and activity of CUGBP1. *J. Biol. Chem.* 276: 7820-7826.

Timchenko,N.A., R.Patel, P.Iakova, Z.J.Cai, L.Quan, and L.T.Timchenko. 2004. Overexpression of CUG triplet repeat-binding protein, CUGBP1, in mice inhibits myogenesis. *J. Biol. Chem.* 279: 13129-13139.

Udd,B., G.Meola, R.Krahe, C.Thornton, L.P.Ranum, G.Bassez, W.Kress, B.Schoser, and R.Moxley. 2006. 140th ENMC International Workshop: Myotonic Dystrophy DM2/PROMM and other myotonic dystrophies with guidelines on management. *Neuromuscul. Disord.* 16: 403-413.

van den Broek,W.J., M.R.Nelen, D.G.Wansink, M.M.Coerwinkel, R.H.te, P.J.Groenen, and B.Wieringa. 2002. Somatic expansion behaviour of the (CTG)n repeat in myotonic dystrophy knock-in mice is differentially affected by Msh3 and Msh6 mismatch-repair proteins. *Hum. Mol. Genet.* 11: 191-198.

van Montfoort,A.P., J.P.Geraedts, J.C.Dumoulin, A.P.Stassen, J.L.Evers, and T.A.Ayoubi. 2008. Differential gene expression in cumulus cells as a prognostic indicator of embryo viability: a microarray analysis. *Mol. Hum. Reprod.* 14: 157-168.

Van,B.J., P.Davis, and S.Alexander. 2000. Differential mitochondrial distribution in human pronuclear embryos leads to disproportionate inheritance between blastomeres: relationship to microtubular organization, ATP content and competence. *Hum. Reprod.* 15: 2621-2633.

Van de Velde H., V, A.De Vos, K.Sermon, C.Staessen, M.De Rycke, E.Van Assche, W.Lissens, M.Vandervorst, H.Van Ranst, I.Liebaers, and A.Van Steirteghem. 2000. Embryo implantation after biopsy of one or two cells from cleavage-stage embryos with a view to preimplantation genetic diagnosis. *Prenat. Diagn.* 20: 1030-1037.

Vandervors,M., C.Staessen, K.Sermon, A.De Vos, d.Van, V, E.Van Assche, M.Bonduelle, A.Vanderfaellie, W.Lissens, H.Tournaye, P.Devroey, A.Van Steirteghem, and I.Liebaers. 2000. The Brussels' experience of more than 5 years of clinical preimplantation genetic diagnosis. *Hum. Reprod. Update.* 6: 364-373.

Vandesompele,J., P.K.De, F.Pattyn, B.Poppe, R.N.Van, P.A.De, and F.Speleman. 2002. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol.* 3: RESEARCH0034.

Verkerk,A.J., M.Pieretti, J.S.Sutcliffe, Y.H.Fu, D.P.Kuhl, A.Pizzuti, O.Reiner, S.Richards, M.F.Victoria, F.P.Zhang, *et al*. . 1991. Identification of a gene (FMR-1) containing a CGG repeat coincident with a breakpoint cluster region exhibiting length variation in fragile X syndrome. *Cell* 65: 905-914.

Verlinsky,Y. and A.Kuliev. 2000. Normal and abnormal human preimplantation development in relation to preimplantation genetic diagnosis and establishment of embryonic stem cells in: *An illustrated textbook and reference for clinicians: An atlas of PGD*. Parthenon Publishing.

Verpoest,W., M.De Rademaeker, K.Sermon, M.De Rycke, S.Seneca, E.Papanikolaou, C.Spits, L.Van Landuyt, E.J.Van der, P.Haentjens, P.Devroey, and I.Liebaers. 2008. Real and expected delivery rates of patients with myotonic dystrophy undergoing intracytoplasmic sperm injection and preimplantation genetic diagnosis. *Hum. Reprod.* 23: 1654-1660.

Walker,M.C., K.E.Murphy, S.Pan, Q.Yang, and S.W.Wen. 2004. Adverse maternal outcomes in multifetal pregnancies. *BJOG.* 111: 1294-1296.

Wang,G.S., D.L.Kearney, B.M.De, G.Taffet, and T.A.Cooper. 2007. Elevation of RNA-binding protein CUGBP1 is an early event in an inducible heart-specific mouse model of myotonic dystrophy. *J. Clin. Invest* 117: 2802-2811.

Warner,J.P., L.H.Barron, D.Goudie, K.Kelly, D.Dow, D.R.Fitzpatrick, and D.J.Brock. 1996. A general method for the detection of large CAG repeat expansions by fluorescent PCR. *J. Med. Genet.* 33: 1022-1026.

Wassarman,P.M. and R.A.Kinloch. 1992. Gene expression during oogenesis in mice. *Mutat. Res.* 296: 3-15.

Wells,D., S.Alfarawati, and E.Fragouli. 2008. Use of comprehensive chromosomal screening for embryo assessment: microarrays and CGH. *Mol. Hum. Reprod.*14 (12): 703-10

Wells,D., M.G.Bermudez, N.Steuerwald, H.E.Malter, A.R.Thornhill, and J.Cohen. 2005a. Association of abnormal morphology and altered gene expression in human preimplantation embryos. *Fertil. Steril.* 84: 343-355.

Wells,D., M.G.Bermudez, N.Steuerwald, A.R.Thornhill, D.L.Walker, H.Malter, J.D.Delhanty, and J.Cohen. 2005b. Expression of genes regulating chromosome segregation, the cell cycle and apoptosis during human preimplantation development. *Hum. Reprod.*20 (5): 1339-48

Wells,D. and J.D.Delhanty. 2000. Comprehensive chromosomal analysis of human preimplantation embryos using whole genome amplification and single cell comparative genomic hybridization. *Mol. Hum. Reprod.* 6: 1055-1062.

Wells,D., T.Escudero, B.Levy, K.Hirschhorn, J.D.Delhanty, and S.Munne. 2002. First clinical application of comparative genomic hybridization and polar body testing for preimplantation genetic diagnosis of aneuploidy. *Fertil. Steril.* 78: 543-549.

Wells,D. and J.K.Sherlock. 1998. Strategies for preimplantation genetic diagnosis of single gene disorders by DNA amplification. *Prenat. Diagn.* 18: 1389-1401.

Wells,D., J.K.Sherlock, A.H.Handyside, and J.D.Delhanty. 1999. Detailed chromosomal and molecular genetic analysis of single cells by whole genome amplification and comparative genomic hybridisation. *Nucleic Acids Res.* 27: 1214-1218.

Wheeler,T.M. and C.A.Thornton. 2007. Myotonic dystrophy: RNA-mediated muscle disease. *Curr. Opin. Neurol.* 20: 572-576.

Wheeler,V.C., F.Persichetti, S.M.McNeil, J.S.Mysore, S.S.Mysore, M.E.MacDonald, R.H.Myers, J.F.Gusella, and N.S.Wexler. 2007. Factors associated with HD CAG repeat instability in Huntington disease. *J. Med. Genet.* 44: 695-701.

Willems,E., I.Mateizel, C.Kemp, G.Cauffman, K.Sermon, and L.Leyns. 2006. Selection of reference genes in mouse embryos and in differentiating human and mouse ES cells. *Int. J. Dev. Biol.* 50: 627-635.

Wilton,L., R.Williamson, J.McBain, D.Edgar, and L.Voullaire. 2001. Birth of a healthy infant after preimplantation confirmation of euploidy by comparative genomic hybridization. *N. Engl. J. Med.* 345: 1537-1541.

Wong,L.J., T.Ashizawa, D.G.Monckton, C.T.Caskey, and C.S.Richards. 1995. Somatic heterogeneity of the CTG repeat in myotonic dystrophy is age and size dependent. *Am. J. Hum. Genet.* 56: 114-122.

Yan,C., P.Wang, J.DeMayo, F.J.DeMayo, J.A.Elvin, C.Carino, S.V.Prasad, S.S.Skinner, B.S.Dunbar, J.L.Dube, A.J.Celeste, and M.M.Matzuk. 2001. Synergistic roles of bone morphogenetic protein 15 and growth differentiation factor 9 in ovarian function. *Mol. Endocrinol.* 15: 854-866.

Yang,J. and C.H.Freudenreich. 2007. Haploinsufficiency of yeast FEN1 causes instability of expanded CAG/CTG tracts in a length-dependent manner. *Gene* 393: 110-115.

Yang,Z., R.Lau, J.L.Marcadier, D.Chitayat, and C.E.Pearson. 2003. Replication inhibitors modulate instability of an expanded trinucleotide repeat at the myotonic dystrophy type 1 disease locus in human cells. *Am. J. Hum. Genet.* 73: 1092-1105.

Yang-Feng,T.L., U.Francke, and A.Ullrich. 1985. Gene for human insulin receptor: localization to site on chromosome 19 involved in pre-B-cell leukemia. *Science* 228: 728-731.

Yotova,V., D.Labuda, E.Zietkiewicz, D.Gehl, A.Lovell, J.F.Lefebvre, S.Bourgeois, E.Lemieux-Blanchard, M.Labuda, H.Vezina, L.Houde, M.Tremblay, B.Toupance, E.Heyer, T.J.Hudson, and C.Laberge. 2005. Anatomy of a founder effect: myotonic dystrophy in Northeastern Quebec. *Hum. Genet.* 117: 177-187.

Zaki,M., P.A.Boyd, L.Impey, A.Roberts, and P.Chamberlain. 2007. Congenital myotonic dystrophy: prenatal ultrasound findings and pregnancy outcome. *Ultrasound Obstet. Gynecol.*29: 284-288.

Zatz,M., A.Cerqueira, M.Vainzof, and M.R.Passos-Bueno. 1997. Segregation distortion of the CTG repeats at the myotonic dystrophy (DM) locus: new data from Brazilian DM families. *J. Med. Genet.* 34: 790-791.

Zatz,M., M.R.Passos-Bueno, A.Cerqueira, S.K.Marie, M.Vainzof, and R.C.Pavanello. 1995. Analysis of the CTG repeat in skeletal muscle of young and adult myotonic dystrophy patients: when does the expansion occur? *Hum. Mol. Genet.* 4: 401-406.

Zeesman,S., L.Zwaigenbaum, D.T.Whelan, R.J.Hagerman, F.Tassone, and S.A.Taylor. 2004. Paternal transmission of fragile X syndrome. *Am. J. Med. Genet. A* 129A: 184-189.

Zhang,P., M.Dixon, M.Zucchelli, F.Hambiliki, L.Levkov, O.Hovatta, and J.Kere. 2008. Expression analysis of the NLRP gene family suggests a role in human preimplantation development. *PLoS. ONE.* 3: e2755.

Zhang,X., N.Jafari, R.B.Barnes, E.Confino, M.Milad, and R.R.Kazer. 2005. Studies of gene expression in human cumulus cells indicate pentraxin 3 as a possible marker for oocyte quality. *Fertil. Steril.* 83 Suppl 1: 1169-1179.

Zheng,P., B.Patel, M.McMenamin, S.E.Reddy, A.M.Paprocki, R.D.Schramm, and K.E.Latham. 2004. The primate embryo gene expression resource: a novel resource to facilitate rapid analysis of gene expression patterns in non-human primate oocytes and preimplantation stage embryos. *Biol. Reprod.* 70: 1411-1418.

Zhu,J., F.He, S.Song, J.Wang, and J.Yu. 2008. How many human genes can be defined as housekeeping with current expression data? *BMC. Genomics* 9: 172.

Ziyyat,A., B.Lassalle, J.Testart, P.Briot, E.Amar, C.Finaz, and A.Lefevre. 1999. Flow cytometry isolation and reverse transcriptase-polymerase chain reaction characterization of human round spermatids in infertile patients. *Hum. Reprod.* 14: 379-387.

Zollner,S., X.Wen, N.A.Hanchard, M.A.Herbert, C.Ober, and J.K.Pritchard. 2004. Evidence for extensive transmission distortion in the human genome. *Am. J. Hum. Genet.* 74: 62-72.

Zuhlke,C., J.Atici, L.Martorell, U.Gembruch, M.Kohl, W.Gopel, and E.Schwinger. 2000. Rapid detection of expansions by PCR and non-radioactive hybridization: application for prenatal diagnosis of myotonic dystrophy. *Prenat. Diagn.* 20: 66-69.

Zunz,E., D.Abeliovich, G.J.Halpern, N.Magal, and M.Shohat. 2004. Myotonic dystrophy--no evidence for preferential transmission of the mutated allele: a prenatal analysis. *Am. J. Med. Genet.* 127A: 50-53.

A1. Appendix 1

A1.1 Chemicals

Most chemicals were supplied by Sigma® Chemical company and BDH (later merged with VWR International) and were of Molecular Biology (for Sigma) or AnalaR Grade (BDH), unless otherwise indicated. The molecular formula or abbreviation for each chemical is shown below.

SIGMA® Chemical Company:

Agarose Type I: low EEO, Bovine serum albumin (BSA), Dimethyl sulfoxide >99.9% (DMSO), DL-Dithiothreitol (DTT) for molecular biology, minimum 99% titration, ethylenediaminetetra-acetic acid anhydrous 99% (EDTA), gelatine from porcine skin type A, glucose (anhydrous), Igepal CA-630, mineral oil, phenol red sodium salt, phosphate buffered saline (PBS), sodium bicarbonate (NaHCO3), sodium chloride (NaCl), sodium dodecyl sulfate 10% solution 0.2µm filtered (SDS), sodium phosphate monobasic 99.0% (NaH2PO4), Tricine

VWR International:

Ethanol 99.7-100% v/v, formaldehyde, glycerol, orthoboric acid, potassium hydroxide (KOH), pottasium chloride (KCl), silver nitrate, sodium borohydride (BNaH4), sodium hydrogen carbonate (NaHCO3), sodium hydrogen orthophosphate, sodium hydroxide pellets (NaOH), potassium hydroxide pellets (KOH), sodium thiosulphate pentahydrate (Na2S2O3 5H2O), Tris (hydroxymethyl) methylamine (TRIS)

A1.2 Solutions

All solutions were prepared with deionized water and sterilized by autoclaving at 121°C, 1 Bar for 30 minutes (Swiftlock compact autoclave, Astell Scientific Ltd).

A1.2.1 DNA extraction

TKM1

10 mM Tris-HCl (pH 7.6), 10 mM KCl, 10 mM MgCl2, 2 mM EDTA (pH 8.0). Autoclaved and stored at room temperature.

TKM2

10 mM Tris-HCl (pH 7.6), 10 mM KCl, 10 mM MgCl2, 0.4 M NaCI, 2 mM EDTA (pH 8.0). Autoclaved and stored at room temperature.

10x TE

10mM Tris-HCl (pH 7.4); 0.1M EDTA (pH 8.0). Autoclaved and stored at room temperature.

6M NaCl

292.2g of NaCl dissolved in 800ml of distilled H2O

10% SDS

100g of electrophoresis-grade SDS dissolved in 900ml of H2O (may need to heat to 68°C). Adjust the pH to 7.2 by adding a few drops of concentrated hydrochloric acid (HCl).

A1.2.2 Lymphocyte isolation

0.9% NaCl

9 g NaCl dissolved in 1000ml H_2O. Autoclaved and stored at room temperature.

A1.2.3 Single cell isolation/lysis

Dissociation buffer (DB)

0.8% NaCl, 0.02% KCl, 0.005% NaH_2PO_4, 0.1% Glucose, 0.1% EDTA, 0.1% $NaHCO_3$, 0.01% Phenol Red

PK lysis buffer

1.25µg/µl proteinase K, 17.5µM sodium dodecyl sulphate

1M NaOH

8g NaOH were dissolved in 200ml distilled H_20. The solution was sterilized by autoclaving and stored at 4°C

1M KOH

11.2g KOH dissolved in 200ml distilled H_20. The solution was sterilized by autoclaving and stored at 4°C

ALB lysis buffer

200mM NaOH or KOH, 50mM DTT

DTT aliquots of 0.007g each (molecular weight: 154.25) were weighed into sterile 1.5ml PCR tubes and stored at 4°C. The lysis buffer was freshly made by adding 200ml of the prepared 1M NaOH or KOH solution to the DTT aliquot to use and adjusting to the final volume of 1ml with nuclease-free water. The volumes of NaOH/KOH and nuclease-free water varied accordingly depending on the amount of DTT in each aliquot.

The freshly made ALB lysis buffer was stored at 4°C and was used for up to two consecutive days.

Tricine (200mM)

0.358g of Tricine was dissolved in 10ml distilled H2O and passed through a 0.2µm sterile filter (Sartorius Minisart®, UK). The solution was aliquoted into 0.5ml PCR tubes, 200µl each, and stored at -20°C.

A1.2.4 Electrophoresis

TBE (10x)

90mM Tris-HCl, 90mM boric acid, 2mM EDTA (pH 8.0). Autoclaved and stored at room temperature.

Loading buffer for agarose gel

40% sucrose; 0.025%w/v bromophenol blue; 0.025% w/v xylene cyanol

Ethidium bromide (stock solution: 10mg/ml)

Diluted to 0.5 μg/ml in 50ml 1X TBE (agarose gel electrophoresis, section 2.5.4.1).

A1.3 Oligonucleotides

Details for oligonucleotides of tables A1.1 and A1.2 were obtained using Ensembl genome browser website, ensemble version 50 (http://www.ensembl.org) or GDB Human Genome Database for the STR markers, heterozygosity details were from GDB.

Table A1.1: Details of oligonucleotide primers for mutation detection and testing of specific targeted regions (*ACTB, AMXY*)

Primer details		**Modification** (as in section 2.4.1.3)	**Primer binding site Basepairs on chromosome (bp)**		**Product size (bp)**
Chromosome 7					
Beta-actin, ENST00000331789 (august 27th 2008)					
***ACTB* Exon 4/5**					
ACTB Forward	GTTGCTATCCAGGCTGTGCT	HEX™	5534276-5534295		470
ACTB Reverse	CGGATGTCCACGTCACACTT		5534821-5534840		
Chromosome 19					
DMPK gene, Ensembl Sequence ENSG00000104936 (august 27th 2008)					
***DMPK* exon 7/8**					
DMPK7/8Forward	GGAGACCTATGGCAAGATCG	FAM™	50972776-50972795		102
DMPK7/8Reverse	AGCAACCGCTGAATGAAGTC		50972618-50972637		
DMPK exon 15					
DMPK1/Forward	GAACGGGGTCGAAGGGTCCTTGTAGC		50965371-50965398		>122 depending on repeat number
DMPK2/Reverse	CTTCCCAGGCCTGCAGTTTGCCCATC	VIC®	50965226-50965251		
DMPK3/Forward	CAGCTCCAGTCCTGTGATCC		50965448-50965467		241bp with DMPK2
TP-PCR primers					
P2	GAACGGGGCTCGAAGGGTCCTTGTAGCCG	FAM™	50965369-50965397		Peak detected every 3bp
P4CAG	TACGCATCCCAGTTTGAGACGCAGCAGCAGCAGCAGCA				
P3R	TACGCATCCCAGTTTGAGACG				
Chromosomes X-Y					
Amelogenin gene, Ensembl Sequence ENSG00000125363 (Chr:X), ENSG0000099721 (Chr:Y)(august 27th 2008)			Chr: X	Chr: Y	
AMELX-Y/ Forward	ATCAGAGCTTAAACTGGGAAGCTG	NED™	11224997-11225020	6797888-6797911	105 (chr:X)
AMELX-Y/ Reverse	CCCTGGGCTCTGTAAAGAATAGTG		11224915-11224935	6797979-6797999	111 (chr:Y)

Table A1.2: Microsatellite markers used in optimised DM1 PGD protocols for detection of contamination

Microsatellite markers					
Primer details		**Modification**	**Primer binding site Base pairs on chromosome (bp)**	**Product size (bp)**	**Heterozygosity (%)**
Chromosome 19					
APOC2 gene Ensembl sequence ENSG00000213044 (august 27th 2008)					
APOC2 Forward	GGCTACATAGCGAGACTCCATCTCC	FAM™	50141347- 50141371	134-170	85.2
APOC2 Reverse	GGGAGAGGGCAAAGATCGATAAAGC		50141216-50141240		
D19S219 Forward	CAGGAAGCGGAGGTTGCAGTGAG	FAM™	50685602-50685625	152-182	77
D19S219 Reverse	GTGGAATTGCTGGGTGGACTGGT		50685746-50685768		
D19S207 Forward	TGCGGTGTTTGAACCCTCGCTG	HEX™	50995802-50995823	135-157	78.4
D19S207 Reverse	ACTGCACTGCAGCCTGAGTGAC		50995932-50995953		
D19S112 Forward	GCCAGCCATTCAGTCATTTGAAG	NED™	51070821-51070843	120-142	86.3
D19S112 Reverse	CTGAAAGACACGTCACACTGGT		51070929-51070950		
Chromosome 21					
D21S11 Forward	TATGTGAGTCAATTCCCCAAGTGA	FAM™	19476131-19476154	172-264	90
D21S11 Reverse	GTTGTATTAGTCAATGTTCTCCAG		19476331-19476354		
D21S1414F	AAATTAGTGTCTGGCACCCAGTA	FAM™	19476463-19476485	291-370	87.5
D21S1414R	CAATTCCCCAAGTGAATTGCCTTC		19476140-19476163		

Table A1.3: Position of primers for RNA/DNA amplification from *ACTB* and *DMPK* genes. Salmon-coloured regions indicate the position of exons. Yellow sequences represent the primer annealing sites.

***ACTB* (ENSG00000075624): DNA product size: 547bp, RNA product size: 452bp**

5534898 ATGGTGTATCTCTGCCTTACAGATCATGTTTGAGACCTTCAACACCCCAGCCATGTACGT 5534839
5534838 TGCTATCCAGGCTGTGCTATCCCTGTACGCCTCTGGCCGTACCACTGGCATCGTGATGGA 5534779
5534778 CTCCGGTGACGGGGTCACCCACACTGTGCCCATCTACGAGGGGTATGCCCTCCCCCATGC 5534719
5534718 CATCCTGCGTCTGGACCTGGCTGGCCGGGACCTGACTGACTACCTCATGAAGATCCTCAC 5534659
5534658 CGAGCGCGGCTACAGCTTCACCACCACGGCCGAGCGGGAAATCGTGCGTGACATTAAGGA 5534599
5534598 GAAGCTGTGCTACGTCGCCCTGGACTTCGAGCAAGAGATGGCCACGGCTGCTTCCAGCTC 5534539
5534538 CTCCCTGGAGAAGAGCTACGAGCTGCCTGACGGCCAGGTCATCACCATTGGCAATGAGCG 5534479
5534478 GTTCCGCTGCCCTGAGGCACTCTTCCAGCCTTCCTTCCTGGGTGAGTGGAGACTGTCTCC 5534419
5534418 CGGCTCTGCCTGACATGAGGGTTACCCCTCGGGGCTGTGCTGTGGAAGCTAAGTCCTGCC 5534359
5534358 CTCATTTCCCTCTCAGGCATGGAGTCCTGTGGCATCCACGAAACTACCTTCAACTCCATC 5534299
5534298 ATGAAGTGTGACGTGGACATCCGCAAAGACCTGTACGCCAACACAGTGCTGTCTGGCGGC 5534239
5534238 ACCACCATGTACCCTGGCATTGCCGACAGGATGCAGAAGGAGATCACTGCCCTGGCACCC 5534179
5534178 AGCACAATGAAGATCAAGGTGGGTGTCTTTCCTGCCTGAGCTGACCTGGGCAGGTCGGCT 5534119
5534118 GTGGGGTCCTGTGGTGTGTGGGGAGCTGTCACATCCAGGGTCCTCACTGCCTGTCCCCTT 5534059

***DMPK* (ENSG00000104936): DNA product size: 176bp, RNA product size: 99bp**

50973035 AGGGCCCAGAGCTGGTGGGCCCAGAGGGGTGGGCCCAAGCCTCGCTCTGCTCCTTTTGGT 50972976
50972975 CCAGGTGCGGTCGCTGGTGGCTGTGGGCACCCCAGACTACCTGTCCCCCGAGATCCTGCA 50972916
50972915 GGCTGTGGGCGGTGGGCCTGGGACAGGCAGCTACGGGCCCGAGTGTGACTGGTGGGCGCT 50972856
50972855 GGGTGTATTCGCCTATGAAATGTTCTATGGGCAGACGCCCTTCTACGCGGATTCCACGGC 50972796
50972795 GGAGACCTATGGCAAGATCGTCCACTACAAGGTGAGCACGGCCGCAGGGAGACCTGGCCT 50972736
50972735 CTCCCGGTAGGCGCTCCCAGGCTATCGCCTCCTCTCCCTCTGAGCAGGAGCACCTCTCTC 50972676
50972675 TGCCGCTGGTGGACGAAGGGGTCCCTGAGGAGGCTCGAGACTTCATTCAGCGGTTGCTGT 50972616
50972615 GTCCCCCGGAGACACGGCTGGGCCGGGGTGGAGCAGGCGACTTCCGGACACATCCCTTCT 50972556
50972555 TCTTTGGCCTCGACTGGGATGGTCTCCGGGACAGCGTGCCCCCCTTTACACCGGATTTCG 50972496
50972495 AAGGTGCCACCGACACATGCAACTTCGACTTGGTGGAGGACGGGCTCACTGCCATGGTGA 50972436
50972435 GCGGGGGCGGGGTAGGTACCTGTGGCCCCTGCTCGGCTGCGGGAACCTCCCCATGCTCCC 50972376

A2. Appendix 2

Table A2.1: Results from fluorescent PCR analysis for all DM1 patients and their relatives for the CTG repeat region, as well as APOC2 and D19S112 polymorphic markers. Underlined alleles indicate the phase. Exp= expanded allele.

Patient number	CTG repeat (bp)	APOC2 (bp)	D19S112 (bp)
1			
affected female	146/Exp	149/151	130/132
unaffected male	122/122	126/149	130/136
2			
affected female	122/Exp	126/<u>154</u>	128/130
unaffected male	122/138	142/149	117/123
female's father (affected)	170/Exp	150/154	128/130
3			
unaffected female	141/160	127/127	128/132
affected male	122/Exp	150/152	130/132
4			
affected female	122/Exp	150/154	<u>130</u>/134
unaffected male	122/146	127/156	118/136
female's cousin (affected)	181/Exp	151/155	128/130
5			
affected female	146/Exp	151/153	<u>117</u>/124
unaffected male	143/143	147/149	128/128
affected foetus (CVS)	146/Exp	151/153	117/128
6			
unaffected female	154/178	126/143	126/128
affected male	142/Exp	<u>151</u>/153	<u>128</u>/130
affected foetus (CVS)	178/Exp	143/151	128/130
7			
affected female	149/Exp	127/<u>143</u>	117/<u>132</u>
unaffected male	122/149	135/154	117/127
female's father (unaffected)	136/136	143/147	117/132
female's uncle (affected)	122/Exp	143/154	123/132
8			
affected female	122/Exp	<u>135</u>/149	117/<u>128</u>
unaffected male	122/146	127/147	117/134
female's mother (affected)	152/Exp	135/149	128/128
female's father (unaffected)	123/150	149/152	117/133
female's brother (affected)	150/Exp	135/149	129/133
male's mother (unaffected)	122/122	127/129	117/125
male's father (unaffected)	146/146	148/156	129/134

Patient number	CTG repeat (bp)	APOC2 (bp)	D19S112 (bp)
9			
affected female	122/Exp	127/150	117/132
unaffected male	122/149	150/152	128/132
female's mother (unaffected)	122/145	127/152	117/138
female's father (affected)	145/Exp	148/150	132/134
female's brother (affected)	122/Exp	127/150	117/132
female's cousin (affected)	139/Exp	127/150	132/140
10			
affected female	145/Exp	148/152	130/134
unaffected male	122/122	127/154	132/136
affected son	122/Exp	147/154	130/132
11			
affected female	139/Exp	149/149	125/130
unaffected male	122/142	127/151	128/130
12			
unaffected female/ egg donor	122/122	150/152	130/136
affected male	122/Exp	143/151	123/128
male's mother (unaffected)	122/122	147/151	123/134
male's brother (unaffected)	122/145	147/153	117/134
13			
affected female	122/Exp	135/153	130/130
unaffected male	122/142	153/153	117/128
female's sister (affected)	139/Exp	153/153	130/130
14			
affected female	122/Exp	127/150	130/130
unaffected male	122/122	149/153	132/134
female's mother (unaffected)	122/122	127/150	130/130
15			
affected female	122/Exp	128/152	130/132
unaffected male	122/137	143/150	117/138
female's father (affected)	176/Exp	128/135	128/130
female's grandmother (unaffected)	122/142	152/159	128/128
female's sister (unaffected)	122/176	135/152	128/132
16			
affected female	139/Exp	148/158	129/129
unaffected male	122/139	150/152	127/127
female's father (affected)	122/Exp	148/150	129/136

Patient number	CTG repeat (bp)	APOC2 (bp)	D19S112 (bp)
17			
unaffected female	122/144	150/150	130/137
affected male	170/Exp	150/154	128/130
male's brother (affected)	122/Exp	150/154	118/130
18			
affected female	122/Exp	149/149	128/137
unaffected male	140/143	149/153	117/130
female's mother (unaffected)	122/141	149/149	128/137
female's father (affected)	147/Exp	149/149	118/128
female's sister1 (affected)	122/Exp	149/149	128/137
female's sister2 (affected)	122/Exp	149/149	128/137
female's sister3 (affected)	122/Exp	149/149	128/128
19			
affected female	180/Exp	157/157	128/130
unaffected male	123/148	129/137	130/133
female's mother (unaffected)	123/180	151/157	128/130
female's father (affected)	144/Exp	149/157	130/130
20			
affected female	141/Exp	151/153	118/137
unaffected male	123/141	151/157	129/131
affected daughter	141/Exp	153/157	118/129
21			
affected female	144/Exp	129/151	128/130
unaffected male	122/147	151/155	117/136
female's mother (unaffected)	144/144	129/153	130/134
female's father (affected)	140/Exp	129/151	128/130
22			
affected female	168/Exp	128/144	117/127
unaffected male	137/140	152/155	117/129
female's mother (unaffected)	122/168	144/150	123/127
23			
affected female	122/Exp	151/151	131/131
unaffected male	122/122	147/153	118/118

Table A2.2 Details of PCR conditions for optimised protocols for PGD for DM1 showing concentration of PCR reaction components as well as PCR cycle programmes performed. Protocols 1-5 had clinical application 20mM Tricine was added as part of the ALB lysis (NaOH or KOH/ DTT) in protocols 2-5. Optimisation also required the use of different enzymes, Amplitaq Gold (protocol 1), Expand High Fidelity (protocols 2 and 3), or Expand Long Template (protocols 4 and 5). In addition, DMSO or glycerol were added in protocols 4 and 5 to improve amplification.

Protocol number/ name (lysis)	Reaction Components	Concentration	PCR program		
			Temperature	Time	Number of cycles
1/ DM1/APOC2 (PK/SDS)	DM1Forward primer-FAM (DMPK1)	0.3µM	94°C	12min	1
	DM1 Reverse primer (DMPK2)	0.3µM	96°C	45secs	10
	APOC2 Forward primer- Hex	0.3µM	60°C	45secs	
	APOC2 Reverse	0.3µM	72°C	1min	
	10mM dNTP	0.2mM	96°C	45secs	30
	10x buffer (with 15mM MgCl2)	1x (1.5mM MgCl2)	60°C	45secs	
	Amplitaq Gold 5U/µl	1.5 units	72°C	1min	
	Nuclease-free water	make up to 22µl	72°C	5min	1
2/ DM1/D19S112 (NaOH/DTT)	DM1Forward primer-VIC (DMPK1)	0.3µM	95°C	2min	1
	DM1 Reverse primer (DMPK2)	0.3µM	96°C	15secs	10
	D19S112 Forward primer- NED	0.4µM	60°C	45secs	
	D19S112 Reverse	0.4µM	72°C	45secs	
	dNTP (10mM)	0.2mM	94°C	45secs	30
	10x Hifi buffer II (with 15mM MgCl2)	1x (1.5mM MgCl2)	60°C	45secs	
	Expand High Fidelity enzyme 5U/µl	1.5units	72°C	45secs	
	Tricine (200mM)	20mM	72°C	7min	1
	Nuclease-free water	make up to 21.5µl			

Protocol number (lysis)	Reaction Components	Concentration	PCR program		
			Temperature	Time	Number of cycles
3/ DM1/APOC2/ D19S112 (NaOH/DTT)	DM1Forward primer- VIC (DMPK1)	0.2µM	95°C	2min	1
	DM1 Reverse primer (DMPK2)	0.2µM	96°C	15secs	10
	APOC2 Forward primer- Fam	0.3µM	58°C	45secs	
	APOC2 Reverse	0.3µM	72°C	1min	
	D19S112 Forward primer- NED	0.3µM	94°C	15secs	30
	D19S112 Reverse	0.3µM	58°C	45secs	
	dNTP (10mM)	0.2mM	72°C	**1min**	
	10x Hifi buffer II (with 15mM MgCl2)	1x (1.5mM MgCl2)	72°C	7min	1
	Expand High Fidelity enzyme 5U/µl	1.5units			
	Tricine (200mM)	20mM			
	Nuclease-free water	make up to 21.5µl			
4/ TP-PCR (KOH/DTT)	P2	0.2µM	95°C	2min	1
	P3R	0.2µM	96°C	30secs	46
	P4CAG	0.1µM	65°C	45secs	
	dNTP (10mM)	0.5mM	72°C	1min	
	Expand Long Template Buffer 3 (27.5mM MgCl2)	1x (2.75mM MgCl2)	72°C	5min	1
	Expand Long Template enzyme 5U/µl	2 units			
	DMSO	5%			
	Tricine (200mM)	20mM			
	Nuclease-free water	make up to 21.5µl			

Protocol number	Reaction Components	Concentration	PCR program		
			Temperature	Time	Number of cycles
5/ TP-PCR/DM1/ D19S112 or mTP-PCR (KOH/DTT)	P2FAM	0.6µM	95°C	2min	1
	DMPK2	0.3µM	96°C	40secs	10
	P4CAG	0.2µM	59°C	1min	
	P3R	0.3µM	72°C	1min	
	D19S112F- NED	0.2µM	94°C	40secs	30
	D19S112R	0.2µM	59°C	1min	
	dNTP (10mM)	0.7mM	72°C	1min	
	Expand Long Template Buffer 3 (27.5mM MgCl2)	1x (2.75mM MgCl2)	72°C	7min	1
	Extra MgCl2	0.6mM			
	Expand Long Template enzyme 5U/µl	2 units			
	Glycerol	10%			
	Tricine (200mM)	20mM			
	Nuclease-free water	make up to 21.5µl			
6/	P2FAM	0.3µM	95°C	2min	1
(KOH/DTT)	P4CAG	0.2µM	95°C	30s	10
	P3R	0.3µM	57°C	45s	
	D19S112F- NED	0.2µM	72°C	1min	
	D19S112R	0.2µM	95°C	30secs	36
	dNTP (10mM)	0.7mM	57°C	45s	
	Expand Long Template Buffer 3 (27.5mM MgCl2)	1x (2.75mM MgCl2)	72°C	1min	
	Expand Long Template enzyme 5U/µl	2 units	72°C	7min	
	Glycerol	10%			
	Tricine (200mM)	20mM			
	Nuclease-free water	make up to 21.5µl			

Table A2.3: Detailed analysis of blastomere amplification, allele dropout (ADO) and diagnosis rate from five DM1 PGD cases using protocols 1 and modification 1a.
*: semi/uninformative patients, unknown phase, h: detection of blastomeres showing amplification from one parental genome

Protocol	Patient Number	Number of embryos	Number of blastomeres	Amplification		ADO		Diagnosis
				DMPK	APOC2	DMPK	APOC2	
1. DM/APOC2	1	4	8	7/8	5/8	1/4	0/4	3/4
	6	10	15	10/15	11/15	0/5	1/9	8/10
	7[h]	10	13	9/13	9/13	0/3	0/6	7/10
	8*	6	12	12/12	10/12	n/a	2/10	1/6
1a. DM/APOC2 split	2	4	6	5/6	3/6	n/a	1/3	3/4

Table A2.4: Detailed analysis of blastomere amplification, allele dropout (ADO) and diagnosis rate from two DM1 PGD cases using protocol 2 for diagnosis,
*: semi/uninformative patients, unknown phase

Protocol	Patient Number	Number of embryos	Number of blastomeres	Amplification		ADO		Diagnosis
				DMPK	D19S112	DMPK	D19S112	
2. DM/D19S112	4	7	14	13/14	13/14	0/3	1/13	6/7
	9*	4	10	8/10	7/10	n/a	n/a	2/4

Table A2.5: Detailed analysis of blastomere amplification, allele dropout (ADO) and diagnosis rate from eleven DM1 PGD cases using protocol 3 for diagnosis, *: semi/uninformative patients, unknown phase, †: diagnosis using two different protocols, h: blastomeres with amplification from one parental genome, H: embryo where both of the biopsied cells indicated amplification from one parental genome only

Protocol	Patient Number/Cycle	Number of embryos	Number of blastomeres	Amplification			ADO			Diagnosis
				DMPK	APOC2	D19S112	DMPK	APOC2	D19S112	
3. DM/APOC2/ D19S112	10	4	5	5/5	5/5	5/5	0/3	0/4	0/4	3/4
	11/1[h]	12	22	21/22	20/22	20/22	0/4	1/16	0/16	8/12
	12[h]	3	6	6/6	6/6	6/6	n/a	n/a	0/2	1/3
	8*[†]	5	8	7/8	7/8	7/8	0/2	0/7	0/2	5/5
	11/2[h]	15	32	27/32	25/32	25/32	0/14	0/17	0/17	12/15
	15	6	7	6/7	5/7	5/7	n/a	0/2	0/1	1/6 [3H]
	19/1	2	4	3/4	3/4	3/4	0/3	0/3	0/3	2/2
	22/1[h]	7	12	11/12	11/12	11/12	0/3	0/10	0/6	7/7
	19/2[h]	5	10	10/10	10/10	10/10	0/7	0/9	0/9	5/5
	22/2	5	7	5/7	6/7	6/7	0/2	0/5	0/5	4/5
	19/3[h]	5	9	9/9	9/9	9/9	0/5	0/7	0/5	4/5 [1H]

Table A2.6: Detailed analysis of blastomere amplification, allele dropout (ADO) and diagnosis rate from five DM1 PGD cases using protocol 4. TP-PCR amplification was scored for non-122 homozygous samples *: semi/uninformative patients, unknown phase, †: diagnosis using two different protocols

Protocol	Patient Number/Cycle	Number of embryos	Number of blastomeres	Amplification	Diagnosis
				TPPCR	
4. TPPCR	9/2*	8	16	9/9	5/8
	13/1*	4	10	9/10	4/4
	8/2*†	5	4^{TPPCR}	3/4	5/5
	13/2*	10	19	12/15	7/10
	16/1	10	18	13/18	9/10

Table A2.7: Detailed analysis of blastomere amplification, allele dropout (ADO) and diagnosis rate from six DM1 PGD cases using protocol 5 for diagnosis, h: blastomeres with amplification from one parental genome, H: embryo where both of the biopsied cells indicated amplification from one parental genome only, *: semi/uninformative patients, unknown phase

Protocol	Patient Number/Cycle	Number of embryos	Number of blastomeres	Amplification			ADO		Diagnosis
				TPPCR	DMPK	D19S112	DMPK	D19S112	
5. TPPCR/DM1/ D19S112	$13/3^{*h}$	8	17	6/6	14/17	14/17	0/2	0/10	$5/8^{3H}$
	20/1	4	8	7/8	6/8	7/8	0/2	0/6	3/4
	$21/1^{h}$	7	13	12/13	9/9**	12/13	n/a	0/8	$5/7^{1H}$
	8/3*	3	6	4/4	6/6	6/6	1/2	0/4	3/3
	23/1	3	6	6/6	6/6	6/6	n/a	0/6	3/3
	23/2	3	10	n/a	7/10	7/10	n/a	0/6	3/3

A3. Appendix 3

Table A3.1: Summary of the fifty genes with highest level of expression in the human MII oocyte

Gene_ Symbol	Gene_Name	Panther_Process	Entrez Gene ID	Celera Gene ID
FTL	ferritin, light polypeptide	Transport|Cation transport;Homeostasis|Other homeostasis activities|Ion transport	2512	hCG39405.3
DNMT1	DNA (cytosine-5-)-methyltransferase 1	DNA metabolism	1786	hCG28474.3
Unassigned	Unassigned	Chromatin packaging and remodeling		hCG2000758
CKS1B	CDC28 protein kinase regulatory subunit 1B	Unclassified	1163	hCG1988891.1| hCG1739274.3| hCG40061.4|hC G15521.3
FLJ40448	Unassigned	Unclassified	339059	
FTL	ferritin, light polypeptide	Cation transport;Homeostasis| Ion transport	2512	hCG39405.3
GPR103	G protein-coupled receptor 103	Signal transduction|G-protein mediated signaling	84109	
Unassigned	Unassigned	Unclassified		hCG2040258.1
RPLP1	ribosomal protein, large, P1	Protein biosynthesis	6176	hCG38799.3
FAM44B	family with sequence similarity 44, member B	Unclassified	91272	hCG41131.4
SH3KBP1 |SMC5L1	SH3-domain kinase binding protein 1|SMC5 structural maintenance of chromosomes 5-like 1 (yeast)	DNA repair|Biological process unclassified|DNA metabolism|		
HNRPA1	heterogeneous nuclear ribonucleoprotein A1	Pre-mRNA processing|mRNA splicing	3178	
LOC400500 |Unassigned	Unassigned	Unclassified	400500	hCG1814062.2
Unassigned	Unassigned	Unclassified		hCG1788212.3
RPL7A	ribosomal protein L7a	Protein biosynthesis	6130	hCG2032998.1| hCG17890.2
RPL7A	ribosomal protein L7a	Protein biosynthesis	6130	hCG2001684
PCNA	proliferating cell nuclear antigen	Unclassified	5111	hCG39115.3
Unassigned	Unassigned	Intracellular protein traffic|Chromosome segregation|Cell structure|Cell motility		hCG1995887.1
Unassigned	Unassigned	Unclassified		hCG2011918
UNG	uracil-DNA glycosylase	DNA repair|Carbohydrate metabolism;Nucleoside, nucleotide and nucleic acid metabolism	7374	hCG38494.3
Unassigned	Unassigned	Protein phosphorylation|Cell cycle control|Mitosis		hCG39453.2
STELLAR |DPPA3	developmental pluripotency associated 3	Unclassified		hCG1659192.3 |hCG2013511.1
H3F3B	H3 histone, family 3B (H3.3B)	Chromatin packaging and remodeling	3021	hCG1989319.1
PTTG3	pituitary tumor-transforming 3	DNA repair|mRNA transcription regulation|Cell cycle control|Chromosome segregation|Oncogene	26255	
PAIP1	poly(A) binding protein interacting protein 1	Protein Biosynthesis	10605	hCG1751685.2
Unassigned	Unassigned	Unclassified		hCG1820938.2
Unassigned	Unassigned	Unclassified		hCG1820954.2
Unassigned	Unassigned	Intracellular protein traffic|Chromosome segregation|Cell structure|Cell motility		hCG1773636.2
C3orf34	chromosome 3 open reading frame 34	Unclassified	84984	
Unassigned	Unassigned	Unclassified		
Unassigned	Unassigned	Translational regulation		
Unassigned	Unassigned	Unclassified		hCG18484.4
Unassigned	Unassigned	Unclassified		hCG1820573.1
Unassigned	Unassigned	Proteolysis		hCG1993742.1
Unassigned	Unassigned	Proteolysis		hCG1643561.2
DPPA3	developmental pluripotency associated 3	Unclassified	359787	hCG1659192.3
TMSB4X	thymosin, beta 4, X-linked	Unclassified	7114	hCG1646598.4
UBB	ubiquitin B	Proteolysis|Protein metabolism and modification	7314	hCG1998947
Unassigned	Unassigned	Protein Biosynthesis		hCG1789827.2
TPRXL	tetra-peptide repeat homeobox-like	Unclassified	348825	hCG2042888
RPL7A	ribosomal protein L7a	Protein biosynthesis	6130	hCG2032998.1 |hCG2028724.1
EEF1A1	eukaryotic translation elongation factor	Biological process		hCG2033271.2

|***DKFZP434F0318***	1 alpha 1	unclassified|Translational regulation|Protein metabolism and modification		
Unassigned	Unassigned	Protein Biosynthesis		hCG1820440.2
DPPA5	developmental pluripotency associated 5	Unclassified	340168	hCG22904.2
SKP2	S-phase kinase-associated protein 2 (p45)	Proteolysis;Oncogenesis|Protein metabolism and modification	6502	hCG36893.3
MORF4L1	mortality factor 4 like 1	mRNA transcription regulation;Developmental processes	10933	hCG2005375
NALP4	NACHT, leucine rich repeat and PYD containing 4	Unclassified	147945	hCG1733040.2
UHRF1	ubiquitin-like, containing PHD and RING finger domains, 1	Nucleoside, nucleotide and nucleic acid metabolism|Other cell cycle process;Cell proliferation and differentiation|Nucleoside, nucleotide and nucleic acid transport;Transport	29128	hCG23497.3 |hCG23738.3
GTF2B	general transcription factor IIB	|mRNA transcription initiation; mRNA transcription regulation|	2959	
GDF9	growth differentiation factor 9	Gametogenesis|Developmental processes|Oogenesis	2661	hCG24129.2
TPT1	tumor protein, translationally-controlled 1	Immunity and defense	7178	hCG32792.2
LOC440055	Unassigned	Protein biosynthesis	440055	hCG27404.2
CDK7	cyclin-dependent kinase 7 (MO15 homolog, Xenopus laevis, cdk-activating kinase)	Cell cycle control|Protein phosphorylation;Cell cycle|Protein metabolism and modification|	1022	hCG1988840
EEF1A1	eukaryotic translation elongation factor 1 alpha 1	Translational regulation|Protein metabolism and modification	1915	hCG2033271.2 |hCG28097.4 |hCG1640413.5

Table A3.2: Summary of the fifty genes with highest level of expression in the human blastocyst

Gene_Symbol	Gene_Name	Panther_Process	Entrez Gene ID	Celera Gene ID
LOC440085	Unassigned	Cell proliferation and differentiation	440085	hCG2003508.1| hCG26572.3
RPL10A	ribosomal protein L10a	Protein metabolism and modification	4736	hCG1787790.1
Unassigned	Unassigned	Protein biosynthesis		hCG2000392.1
RPL37P6	ribosomal protein L37 pseudogene 6	Protein biosynthesis	346950	hCG39750.2
Unassigned	Unassigned	Protein biosynthesis		hCG2043433
RPS2	ribosomal protein S2	Protein biosynthesis	6187	hCG1990006|hCG1983409| hCG2018618.2|hCG2016250
RPS3A	ribosomal protein S3A	Protein biosynthesis	6189	hCG33299.3
RPS4X	ribosomal protein S4, X-linked	Protein biosynthesis	6191	hCG18634.3
Unassigned	Unassigned	Protein folding|Nuclear transport|Immunity and defense		hCG1794401.2
RPL12	ribosomal protein L12	Protein biosynthesis	6136	
RPS11	ribosomal protein S11	Protein biosynthesis	6205	hCG16209.3
RPL26	ribosomal protein L26	Protein biosynthesis	6154	hCG1985370
FLJ40448	hypothetical protein FLJ40448	Biological process unclassified	339059	
RPLP1	ribosomal protein, large, P1	Protein biosynthesis	6176	hCG38799.3
Unassigned	Unassigned	Protein folding|Nuclear transport|Immunity and defense		hCG32230.3
LOC401896	Unassigned	Protein biosynthesis|Protein metabolism and modification	401896	hCG1775736.1
PABPC1	poly(A) binding protein, cytoplasmic 1	mRNA end-processing and stability	26986	hCG15683.3
RPS25	ribosomal protein S25	Unclassified	6230	hCG1641401.2
PRDX1	peroxiredoxin 1	Antioxidation and free radical removal|Immunity and defense	5052	hCG1780053.2

RPL24	ribosomal protein L24	Protein biosynthesis	6152	hCG2023003.1
HSPE1	heat shock 10kDa protein 1 (chaperonin 10)	Protein metabolism and modification	3336	hCG21429.4
Unassigned	Unassigned	Protein biosynthesis		hCG1981229
RPL7*|*LOC441896*|*EIF3S10	ribosomal protein L7|eukaryotic translation initiation factor 3, subunit 10 theta, 150/170kDa	Biological process unclassified|Protein biosynthesis;Protein metabolism and modification|Translational regulation		hCG31916.3|hCG17114.3|hCG1783090.1|hCG1983988|hCG1640398.3
GPR103	G protein-coupled receptor 103	Cell surface receptor mediated signal transduction|Signal transduction|G-protein mediated signaling	84109	
ENO1	enolase 1, (alpha)	Glycolysis|Carbohydrate metabolism	2023	hCG22399.3
HNRPA1	heterogeneous nuclear ribonucleoprotein A1	Pre-mRNA processing|mRNA splicing	3178	
KRT18	keratin 18	Cell structure and motility	3875	hCG43757.3
LOC440055	Unassigned| Similar to ribosomal protein S12	Protein biosynthesis	440055	hCG27404.2
Unassigned	Unassigned	Unclassified		hCG1820573.1
RPS27A	ribosomal protein S27a	Proteolysis|Protein metabolism and modification	6233	hCG1987923
EEF2	eukaryotic translation elongation factor 2	Protein biosynthesis	1938	hCG23520.3
LOC388339	Unassigned | similar to ribosomal protein S18	Protein biosynthesis	388339	hCG1640711.4
RPS7	ribosomal protein S7	Protein metabolism and modification	6201	hCG31799.1|hCG2043512|hCG1639825.4|hCG1784266.2
RPL7A	ribosomal protein L7a	Protein biosynthesis	6130	hCG2032998.1|hCG17890.2
Unassigned	Unassigned	Unclassified		hCG1816993.1
RPL7A	ribosomal protein L7a	Protein biosynthesis	6130	hCG2001684
RPS2	ribosomal protein S2	Protein biosynthesis|	6187	hCG1990006|hCG2018618.2|hCG2016250
LOC343384	Unassigned | similar to peptidylprolyl isomerase A isoform 1	Protein metabolism and modification|Nuclear transport;Immunity and defense|Protein folding;Intracellular protein traffic	343384	hCG1792358.3
RPL37	ribosomal protein L37	Protein biosynthesis	6167	
RPLP2	ribosomal protein, large, P2	Protein biosynthesis	6181	hCG1778304.2
Unassigned	Unassigned	Protein biosynthesis		hCG22004.2
HSPE1*|*LOC387880	heat shock 10kDa protein 1 (chaperonin 10)	Protein metabolism and modification		hCG2040162|hCG21429.4|hCG26559.2
Unassigned	Unassigned	Protein biosynthesis		hCG1999595.1
Unassigned	Unassigned	Biological process unclassified		hCG1643652.1
LOC401859	Unassigned	Protein metabolism and modification|Nuclear transport;Immunity and defense|Protein folding;Intracellular protein traffic	401859	hCG1655497.4
LOC391062	Unassigned	Protein metabolism and modification|Nuclear transport;Immunity and defense|Protein folding;Intracellular protein traffic	391062	hCG1643204.4

GOLGA8A*| *SLC25A6	golgi autoantigen, golgin subfamily a, 8A|solute carrier family 25 (mitochondrial carrier; adenine nucleotide translocator), member 6	Protein targeting and localization|Nucleoside, nucleotide and nucleic acid transport;Transport|Nucleoside, nucleotide and nucleic acid metabolism|		hCG1746794.2
RPL18	ribosomal protein L18	Protein biosynthesis	6141	hCG2000370.1

Table A3.3: List of 349 high expression oocyte-specific genes. Genes are listed in ascending order by their Celera ID.

hCG14713	hCG16632	hCG1786105	hCG1814486	hCG1993933
hCG14841	hCG16730	hCG1786193	hCG1814527	hCG19952
hCG15000	hCG16811	hCG1786829	hCG1815686	hCG1995942
hCG15050	hCG16828	hCG1786864	hCG1817290	hCG1997847
hCG15108	hCG16929	hCG17871	hCG1817498	hCG2000011
hCG15138	hCG17062	hCG1788005	hCG1817751	hCG2000584
hCG15256	hCG1729956	hCG1788604	hCG1817877	hCG2001986
hCG15364	hCG1733021	hCG1791825	hCG1818446	hCG2003734
hCG15438	hCG17382	hCG1792164	hCG1818459	hCG2003750
hCG15525	hCG1742115	hCG1792234	hCG1820440	hCG20042
hCG15532	hCG1743207	hCG1793655	hCG1820599	hCG20064
hCG15558	hCG1743785	hCG1796067	hCG1821231	hCG2006852
hCG15574	hCG1744908	hCG1803572	hCG18324	hCG2008598
hCG15670	hCG17542	hCG18043	hCG18601	hCG2009388
hCG15959	hCG17601	hCG18066	hCG18988	hCG2009910
hCG16030	hCG17664	hCG1810759	hCG19156	hCG2010233
hCG1639758	hCG1773636	hCG1810859	hCG19212	hCG2010889
hCG1640673	hCG1773884	hCG1810884	hCG19607	hCG2011013
hCG1640820	hCG1775361	hCG1811008	hCG19614	hCG20111
hCG1640936	hCG1776311	hCG1811012	hCG19657	hCG2011580
hCG1642455	hCG1777406	hCG1811035	hCG1979388	hCG2011781
hCG1643224	hCG1777721	hCG1811036	hCG1979495	hCG2012284
hCG1645228	hCG1779020	hCG1811059	hCG1983051	hCG2013331
hCG1645324	hCG1779729	hCG1811092	hCG1983510	hCG2015975
hCG1646184	hCG1779876	hCG1811301	hCG1984799	hCG2016229
hCG1646296	hCG1780614	hCG1811428	hCG1987869	hCG2017381
hCG1648127	hCG1781169	hCG1811512	hCG1988045	hCG2018215
hCG1649960	hCG1781628	hCG1811857	hCG1988995	hCG2020974
hCG1652542	hCG1782268	hCG1811876	hCG1989524	hCG2020975
hCG16537	hCG1784522	hCG1812169	hCG1990670	hCG2021087
hCG1654498	hCG1784573	hCG1812722	hCG1990839	hCG2022610
hCG16610	hCG1784779	hCG1813574	hCG1991018	hCG2026013
hCG16630	hCG1785346	hCG1814062	hCG19911	hCG2026687
hCG16631	hCG1785623	hCG1814125	hCG1991909	hCG2027083

hCG2028192	hCG20893	hCG27946	hCG38627	hCG44065
hCG2029590	hCG20960	hCG28273	hCG38829	hCG95858
hCG2030297	hCG21077	hCG28318	hCG38835	hCG96668
hCG2032408	hCG21309	hCG28377	hCG38864	
hCG2032644	hCG21329	hCG28718	hCG39059	
hCG2033524	hCG21344	hCG28984	hCG39145	
hCG2033819	hCG21358	hCG29469	hCG39179	
hCG2036556	hCG21652	hCG29614	hCG39261	
hCG2036560	hCG21731	hCG30009	hCG39323	
hCG2036573	hCG21983	hCG30288	hCG39347	
hCG2036806	hCG22192	hCG30333	hCG39450	
hCG2036813	hCG22215	hCG31079	hCG39453	
hCG2036869	hCG22390	hCG32235	hCG39463	
hCG2038156	hCG22899	hCG32352	hCG39637	
hCG2038216	hCG23039	hCG32377	hCG39667	
hCG2038584	hCG23318	hCG32610	hCG39684	
hCG2038661	hCG23447	hCG32735	hCG39762	
hCG2038731	hCG23626	hCG32909	hCG39779	
hCG2039068	hCG23667	hCG32918	hCG39788	
hCG2039417	hCG23672	hCG33075	hCG39798	
hCG2039566	hCG23837	hCG33085	hCG39814	
hCG2039875	hCG23972	hCG33087	hCG40002	
hCG2039945	hCG24112	hCG33190	hCG40021	
hCG2039979	hCG24113	hCG33516	hCG401120	
hCG2040243	hCG24129	hCG34128	hCG401169	
hCG2040324	hCG24574	hCG34805	hCG401172	
hCG2040656	hCG24794	hCG34806	hCG401209	
hCG2040899	hCG24796	hCG36755	hCG401218	
hCG2040997	hCG24865	hCG36838	hCG401272	
hCG2041111	hCG24964	hCG36946	hCG40256	
hCG2041254	hCG25031	hCG37128	hCG40279	
hCG2041389	hCG25127	hCG37158	hCG40396	
hCG2041810	hCG25456	hCG37562	hCG40452	
hCG2041922	hCG26491	hCG37727	hCG40463	
hCG2042091	hCG26558	hCG37737	hCG40828	
hCG2042450	hCG26763	hCG37802	hCG40969	
hCG2042571	hCG26948	hCG38014	hCG40983	
hCG2042895	hCG27387	hCG38025	hCG41011	
hCG2042959	hCG27456	hCG38088	hCG41052	
hCG2043425	hCG27671	hCG38094	hCG41358	
hCG2045906	hCG27690	hCG38109	hCG41502	
hCG2045907	hCG27752	hCG38241	hCG41795	
hCG20711	hCG27926	hCG38242	hCG41855	
hCG20715	hCG27927	hCG38309	hCG43352	

Table A3.4: List of 431 high expression blastocyst-specific genes. Genes are listed in ascending order by their Celera ID.

hCG14618	hCG1643162	hCG1742531	hCG1795553	hCG19468
hCG14649	hCG1643175	hCG1743761	hCG1795633	hCG19516
hCG14659	hCG16432	hCG1743779	hCG17996	hCG19609
hCG14677	hCG1643228	hCG1743861	hCG18049	hCG19672
hCG14678	hCG1643231	hCG1745153	hCG1807414	hCG19693
hCG14847	hCG1643466	hCG1746935	hCG18100	hCG1981097
hCG14929	hCG1643663	hCG1748768	hCG1810958	hCG1981800
hCG15413	hCG1643722	hCG1757059	hCG1811060	hCG1984423
hCG15518	hCG1643895	hCG1759133	hCG1811095	hCG1985920
hCG15580	hCG1644263	hCG17638	hCG1811258	hCG1986317
hCG15695	hCG1644378	hCG1770271	hCG1811302	hCG1986432
hCG15708	hCG1644435	hCG17757	hCG1811547	hCG1986580
hCG15752	hCG1644607	hCG1776197	hCG1812757	hCG1987797
hCG15917	hCG1646237	hCG1776475	hCG1812787	hCG1988058
hCG16023	hCG1646720	hCG1776677	hCG1812951	hCG1988300
hCG16097	hCG1647067	hCG1780024	hCG18135	hCG1988320
hCG16292	hCG1651416	hCG1780842	hCG1814536	hCG1988454
hCG16295	hCG1657259	hCG1780948	hCG18153	hCG19906
hCG1639748	hCG1660125	hCG1781103	hCG1817354	hCG1990963
hCG1639843	hCG16678	hCG1781910	hCG1817472	hCG1990983
hCG1639963	hCG16827	hCG1782057	hCG1817599	hCG1991474
hCG1640153	hCG1685830	hCG1782327	hCG1817746	hCG1991560
hCG1640184	hCG16920	hCG1782581	hCG1817985	hCG1992111
hCG1640242	hCG16946	hCG17829	hCG1818367	hCG1992407
hCG1640272	hCG17039	hCG1783391	hCG1818503	hCG1993582
hCG1640390	hCG17108	hCG1784039	hCG1818525	hCG1994498
hCG1640406	hCG17225	hCG1784107	hCG1818651	hCG19946
hCG1640549	hCG17250	hCG1784432	hCG1820431	hCG1996391
hCG1640610	hCG1726843	hCG1784504	hCG1820468	hCG1997251
hCG1640751	hCG1730394	hCG1785265	hCG1820516	hCG1998331
hCG1640879	hCG1730824	hCG1786960	hCG1820586	hCG1998384
hCG1640970	hCG17325	hCG1787379	hCG1820647	hCG1998392
hCG1641138	hCG1735238	hCG1787380	hCG1820745	hCG1999033
hCG1641204	hCG1736101	hCG1787521	hCG1820912	hCG1999172
hCG1641569	hCG1736511	hCG1788236	hCG18278	hCG1999887
hCG1641604	hCG17376	hCG1788612	hCG18435	hCG2002932
hCG1641789	hCG1738619	hCG1789710	hCG18485	hCG2004008
hCG1641902	hCG1739142	hCG1790391	hCG18525	hCG2004157
hCG1642370	hCG1739343	hCG1792076	hCG19108	hCG2004161
hCG1642703	hCG1740373	hCG1794543	hCG19232	hCG2004404
hCG1643070	hCG1741622	hCG1795423	hCG19408	hCG2004932

hCG2005740	hCG2039420	hCG22225	hCG29617	hCG38953
hCG2010765	hCG2039620	hCG22593	hCG30597	hCG38986
hCG2011004	hCG2039995	hCG22755	hCG30600	hCG39037
hCG2011423	hCG2039996	hCG22783	hCG30902	hCG39149
hCG201191	hCG2040013	hCG22878	hCG31372	hCG39157
hCG201245	hCG20401	hCG23101	hCG31406	hCG39228
hCG2013610	hCG2040228	hCG23188	hCG31440	hCG39249
hCG2013819	hCG2040233	hCG23234	hCG31492	hCG39324
hCG20142	hCG2040291	hCG23542	hCG31497	hCG39359
hCG2014502	hCG2040298	hCG23634	hCG31729	hCG39383
hCG2014568	hCG2040351	hCG23676	hCG31749	hCG39398
hCG2014892	hCG2040365	hCG23989	hCG32037	hCG39465
hCG2015268	hCG2040606	hCG24009	hCG32191	hCG39510
hCG2015274	hCG2041332	hCG24101	hCG32473	hCG39512
hCG2016736	hCG2042151	hCG24350	hCG32739	hCG39534
hCG2017435	hCG2042785	hCG24422	hCG32779	hCG39580
hCG2018184	hCG2043046	hCG24698	hCG33003	hCG39636
hCG2019820	hCG2043341	hCG25017	hCG33030	hCG39689
hCG2019888	hCG2043376	hCG25143	hCG33071	hCG39760
hCG2020139	hCG2043377	hCG25185	hCG33191	hCG39777
hCG2020552	hCG2043431	hCG25596	hCG34058	hCG39823
hCG2021371	hCG2043508	hCG25636	hCG34196	hCG39829
hCG2022032	hCG2043539	hCG25780	hCG34454	hCG39882
hCG2022619	hCG2043597	hCG25942	hCG36855	hCG39988
hCG2022772	hCG20459	hCG26002	hCG37044	hCG39991
hCG2025279	hCG20487	hCG26012	hCG37127	hCG40096
hCG2025667	hCG20535	hCG26797	hCG37191	hCG401161
hCG2026171	hCG20599	hCG27168	hCG37261	hCG401308
hCG2026794	hCG20682	hCG27479	hCG37525	hCG40151
hCG2027246	hCG20913	hCG27512	hCG37572	hCG40163
hCG2027322	hCG21032	hCG27612	hCG37613	hCG40270
hCG2027596	hCG21055	hCG27800	hCG37641	hCG40338
hCG20299	hCG21174	hCG27811	hCG37696	hCG40602
hCG2030721	hCG21230	hCG27833	hCG37734	hCG40623
hCG2032547	hCG21336	hCG28013	hCG37853	hCG40703
hCG2032955	hCG21525	hCG28119	hCG37922	hCG40734
hCG2033672	hCG21638	hCG28214	hCG37997	hCG40887
hCG2033702	hCG21682	hCG28314	hCG38021	hCG40991
hCG2036618	hCG21844	hCG28366	hCG38030	hCG41030
hCG2036645	hCG21906	hCG28439	hCG38062	hCG41100
hCG2036753	hCG21985	hCG28483	hCG38480	hCG41139
hCG2038863	hCG21988	hCG28819	hCG38568	hCG41245
hCG2039083	hCG22086	hCG29185	hCG38709	hCG41344
hCG2039386	hCG22165	hCG29302	hCG38806	hCG41785

hCG42023
hCG42708
hCG42867
hCG43349
hCG43758
hCG96709

Table A3.5: Genes with high expression in oocytes and embryos out of the 525 housekeeping genes investigated.

	Oocytes (number of genes)	Blastocysts (number of genes)	Shared genes of high signal (number of genes)
Transcription pre-initiation complex	*CCNH, CDK7, GTF2A2, GTF2B, GTF2E1, GTF2F2, GTF2H2, MNAT1, POLR2F, POLR2H, POLR2K, TAF10, TAF12, TAF4B, TAF5, TAF6, TAF9, TBP* (18)	*CDK7, GTF2A2, GTF2B, GTF2E1, GTF2E2, GTF2F2, POLR2B, POLR2E, POLR2F, POLR2G, POLR2H, POLR2I, POLR2K, TAF10, TAF12, TAF6, TAF7, TAF9, TBP* (19)	*CDK7, GTF2A2, GTF2B, GTF2E1, GTF2F2, POLR2F, POLR2H, POLR2K, TAF10, TAF12, TAF6, TAF9, TBP* (13)
Transcription elongation complex	*CDK9, SSRP1, TCEB1, WHSC2* (4)	*CDK9, RDBP, SSRP1, TCEA1, TCEB1, TCEB2* (6)	*CDK9, SSRP1, TCEB1* (3)
Essential splicing factor	*CCAR1, DNAJC8, FLJ10292, FUS, MAGOH, NFX1, RBM8A, RNPS1, SF1, SFRS11, SFRS4, SFRS5, SFRS7, SFRS9, U2AF1, YBX1* (16)	*CCAR1, DHX9, DNAJC8, FLJ10292, FUS, MAGOH, RBM8A, RNPS1, SF1, SF4, SFRS1, SFRS11, SFRS2, SFRS3, SFRS4, SFRS5, SFRS6, SFRS7, SFRS9, SRRM1, THOC4, U2AF1, YBX1* (23)	*CCAR1, DNAJC8, FLJ10292, FUS, MAGOH, RBM8A, SF1, SFRS11, SFRS4, SFRS5, SFRS7, SFRS9, U2AF1* (13)
hnRNP	*HNRPA2B1, HNRPA3|HNRPA3P1, HNRPC, HNRPF, HNRPK, HNRPM, PCBP1, RBMX* (8)	*HNRPA1, HNRPA2B1, HNRPA3|HNRPA3P1, HNRPC, HNRPD, HNRPF, HNRPH1, HNRPK, HNRPM, HNRPR, HNRPU, PTBP1, RBMX* (13)	*HNRPA2B1, HNRPA3|HNRPA3P1, HNRPC, HNRPF, HNRPK, HNRPM, RBMX* (7)
snRNP	*HNRPC, SF3A3, SF3B1, SF3B2, SNRPB, SNRPB2, SNRPD1, SNRPD2, SNRPD3, SNRPE|SNRPEL1, SNRPF|ENO1, SNRPG, TXNL4A, WDR57* (14)	*EFTUD2, HNRPC, LSM2, NHP2L1, SF3A3, SF3B1, SF3B2, SF3B3, SF3B4, SF3B5, SNRPA, SNRPB, SNRPB2, SNRPC, SNRPD1, SNRPD2, SNRPD3, SNRPE|SNRPEL1, SNRPF|ENO1, SNRPG, TXNL4A, WDR57* (22)	*HNRPC, SF3A3, SF3B1, SF3B2, SNRPB, SNRPB2, SNRPD1, SNRPD2, SNRPD3, SNRPE|SNRPEL1, SNRPF|ENO1, SNRPG, TXNL4A, WDR57* (14)
Capping related genes	*NCBP1* (1)	*NCBP2, NCBP1* (2)	*NCBP1* (1)
Cleavage and polyadenylation complex	*CPSF2, CPSF4, CSTF1, CSTF2, NUDT21* (5)	*COLEC12, CPSF2, CPSF3, CPSF4, CSTF1, CSTF2, CSTF3, FLJ12529, NUDT21, PAPOLA* (10)	*CPSF2, CPSF4, CSTF1, CSTF2, NUDT21* (5)
Nuclear Pore Complex	*AAAS, NUP107, NUP133, NUP153, NUP35, NUP37, NUP50, NUP62, NUP85, NUP88, NUP93, NUP98, NUPL2, RAE1, RANBP2* (15)	*AAAS, NUP133, NUP153, NUP188, NUP35, NUP37, NUP50, NUP62, NUP85, NUP93, RANBP2* (11)	*RANBP2, AAAS, NUP133, NUP153, NUP35, NUP37, NUP50, NUP62, NUP85, NUP93* (10)

Translation, initiation, elongation and termination factor	*EEF1A1*, *EEF1B2*, *EEF1D*, *EEF1G*, *EEF2*, *EIF1*, *EIF1AX*|*EIF1AP1*, *EIF2B1*, *EIF2B2*, *EIF2S1*, *EIF3S10*, *EIF3S12*, *EIF3S2*, *EIF3S3*, *EIF3S5*|*LOC339799*, *EIF3S6*, *EIF3S7*, *EIF3S8*, *EIF4A1*, *EIF4A2*, *EIF4E*, *EIF5B*, *WBSCR1* (23)	*EEF2*, *EEF1A1*, *EEF1B2*, *EEF1D*, *EEF1G*, *EIF1*, *EIF1AX*|*EIF1AP1*, *EIF2B1*, *EIF2B2*, *EIF2B3*, *EIF2S1*, *EIF2S2*, *EIF3S1*, *EIF3S10*, *EIF3S12*, *EIF3S2*, *EIF3S3*, *EIF3S4*, *EIF3S5*|*LOC339799*, *EIF3S6*, *EIF3S7*, *EIF3S8*, *EIF3S9*, *EIF4A1*, *EIF4A2*, *EIF4B*, *EIF4E*, *EIF4EBP1*, *EIF4G1*, *EIF5*, *ETF1*, *WBSCR1* (32)	*EEF1A1*, *EEF1D*, *EEF2*, *EIF1*, *EIF1AX*|*EIF1AP1*, *EIF2B1*, *EIF2B2*, *EIF2S1*, *EIF3S10*, *EIF3S12*, *EIF3S2*, *EIF3S3*, *EIF3S5*|*LOC339799*, *EIF3S6*, *EIF3S7*, *EIF3S8*, *EIF4A1*, *EIF4A2*, *EIF4E*, *WBSCR1* (20)
tRNA synthesis	*AARS*, *DARS*, *EPRS*, *GARS*, *NARS*, *QARS* (6)	*AARS*, *DARS*, *EPRS*, *GARS*, *GART*, *KARS*, *LARS*, *MARS*, *NARS*, *QARS*, *RARS*, *SARS*, *TARS*, *VARS*, *WARS*, *YARS* (16)	*AARS*, *DARS*, *EPRS*, *GARS*, *NARS*, *QARS* (6)
Cytosolic ribosome	*C15orf15*, *FAU*, *LOC387907*|*RPS7*, *LOC388654*|*LOC388524*|*LOC387867*|*LAMR1P15*|*RPSA*, *LOC440520*|*RPS27*, *LOC441136*|*RPL35A*, *LOC497661*, *MRPL13*, *RPL10A*, *RPL13*|*LOC388344*, *RPL13A*|*LOC283340*|*RP11-365K22.1*, *RPL14*, *RPL15*, *RPL17*|*LOC390773*, *RPL18*, *RPL18A*, *RPL19*, *RPL23*, *RPL23A*, *RPL24*, *RPL26*, *RPL26L1*, *RPL27*, *RPL27A*, *RPL28*, *RPL29*, *RPL3*|*ZNF114*, *RPL30*, *RPL31*, *RPL35*, *RPL36*, *RPL37A*, *RPL38*, *RPL4*, *RPL41*, *RPL5*, *RPL7A*, *RPL8*, *RPL9*, *RPLP0*, *RPLP1*, *RPLP2*, *RPS11*, *RPS12*, *RPS15*|*LOC401019*, *RPS15A*, *RPS16*, *RPS17*, *RPS18*, *RPS19*, *RPS20*, *RPS21*, *RPS23*, *RPS24*, *RPS25*, *RPS27A*, *RPS28*|*LOC441618*, *RPS29*, *RPS4X*, *RPS5*, *RPS6*, *RPS7*, *RPS8*, *RPS9*, *SLC36A2*|*RPL24*, *UBA52* (66)	*C15orf15*, *FAU*, *LOC388654*|*RPSA*|*LOC388954*|*LOC388122*|*LOC441447*, *LOC440520*|*RPS27*, *LOC441136*|*RPL35A*, *LOC497661*, *MRPL13*, *MRPS12*, *RPL10A*, *RPL11*, *RPL13*|*LOC388344*, *RPL13A*|*LOC283340*|*RP11-365K22.1*, *RPL14*, *RPL15*, *RPL17*|*LOC390773*, *RPL18*, *RPL18A*, *RPL19*, *RPL23*, *RPL23A*, *RPL24*, *RPL26*, *RPL26L1*, *RPL27*, *RPL27A*, *RPL28*, *RPL29*, *RPL3*|*ZNF114*, *RPL30*, *RPL31*, *RPL35*, *RPL36*, *RPL36AL*, *RPL37A*, *RPL38*, *RPL4*, *RPL41*, *RPL5*, *RPL7A*, *RPL8*, *RPL9*, *RPLP0*, *RPLP1*, *RPLP2*, *RPS11*, *RPS12*, *RPS14*, *RPS15*|*LOC401019*, *RPS15A*, *RPS16*, *RPS17*, *RPS18*, *RPS19*, *RPS2*, *RPS20*, *RPS21*, *RPS23*, *RPS24*, *RPS25*, *RPS27A*, *RPS28*|*LOC441618*, *RPS29*, *RPS3*, *RPS4X*, *RPS4Y1*, *RPS5*, *RPS6*, *RPS7*, *RPS8*, *RPS9*, *SLC36A2*|*RPL24*, *UBA52* (72)	*C15orf15*, *FAU*, *LOC388654*|*RPSA*|*LOC388954*|*LOC388122*|*LOC441447*, *LOC440520*|*RPS27*, *LOC441136*|*RPL35A*, *LOC497661*, *MRPL13*, *RPL10A*, *RPL13*|*LOC388344*, *RPL13A*|*LOC283340*|*RP11-365K22.1*, *RPL14*, *RPL15*, *RPL17*|*LOC390773*, *RPL18*, *RPL18A*, *RPL19*, *RPL23*, *RPL23A*, *RPL24*, *RPL26*, *RPL26L1*, *RPL27*, *RPL27A*, *RPL28*, *RPL29*, *RPL3*|*ZNF114*, *RPL30*, *RPL31*, *RPL35*, *RPL36*, *RPL37A*, *RPL38*, *RPL4*, *RPL41*, *RPL5*, *RPL7A*, *RPL8*, *RPL9*, *RPLP0*, *RPLP1*, *RPLP2*, *RPS11*, *RPS12*, *RPS15*|*LOC401019*, *RPS15A*, *RPS16*, *RPS17*, *RPS18*, *RPS19*, *RPS20*, *RPS21*, *RPS23*, *RPS24*, *RPS25*, *RPS27A*, *RPS28*|*LOC441618*, *RPS29*, *RPS4X*, *RPS5*, *RPS6*, *RPS7*, *RPS8*, *RPS9*, *SLC36A2*|*RPL24*, *UBA52* (65)
Ubiquitin mediated proteolysis	*ANAPC10*, *ANAPC11*, *ANAPC4*, *BTRC*, *CDC20*, *CUL1*, *FBXW11*, *RBX1*, *SKP1A*, *SKP2*, *TCEB1*, *UBE2C*, *UBE2D1*, *UBE2D2*, *UBE2D3*, *UBE2R2* (16)	*ANAPC10*, *ANAPC11*, *CDC16*, *RBX1*, *SKP1A*, *SKP2*, *TCEB1*, *TCEB2*, *UBE2C*, *UBE2D1*, *UBE2D2*, *UBE2D3*, *UBE2R2* (13)	*ANAPC10*, *ANAPC11*, *RBX1*, *SKP1A*, *SKP2*, *TCEB1*, *UBE2C*, *UBE2D1*, *UBE2D2*, *UBE2D3*, *UBE2R2* (11)

Proteasome	*PSMA2*, *PSMA3*, *PSMA4*, *PSMA5*, *PSMA7*, *PSMB1*, *PSMB2*, *PSMB3*, *PSMB4*, *PSMB5*, *PSMB6*, *PSMB7*, *PSMC1*, *PSMC2*, *PSMC4*, *PSMC6*, *PSMD1*, *PSMD11*, *PSMD14*, *PSMD2*, *PSMD6*, *PSMD7*, *PSME3* (23)	*PSMA1*, *PSMA2*, *PSMA3*, *PSMA4*, *PSMA5*, *PSMA6*, *PSMA7*, *PSMB1*, *PSMB2*, *PSMB3*, *PSMB4*, *PSMB5*, *PSMB6*, *PSMB7*, *PSMC1*, *PSMC2*, *PSMC3*, *PSMC4*, *PSMC5*, *PSMC6*, *PSMD1*, *PSMD11*, *PSMD14*, *PSMD2*, *PSMD4*, *PSMD6*, *PSMD7*, *PSME2*, *PSME3* (29)	*PSMA2*, *PSMA3*, *PSMA4*, *PSMA5*, *PSMA7*, *PSMB1*, *PSMB2*, *PSMB3*, *PSMB4*, *PSMB5*, *PSMB6*, *PSMB7*, *PSMC1*, *PSMC2*, *PSMC4*, *PSMC6*, *PSMD1*, *PSMD11*, *PSMD14*, *PSMD2*, *PSMD6*, *PSMD7*, *PSME3* (23)
Other common HK genes*, excluding genes shared with other datasets	*ACTB*, *ACTG1*, *ANP32B*, *ARF1*, *ARF4*, *ARPC2*, *ATF4*, *ATP5A1*, *ATP5G1*, *ATP5G3*, *ATP5O*, *ATP6V1F*, *BAT1*, *CALM2*, *CANX*, *CASC3*, *CLN5*, *CLTA*, *COX4I1*, *COX6A1*, *COX7A2*, *COX7C*, *COX8A*, *CSNK2B*, *CSTB*, *CYB5R3*, *DAZAP2*, *DDX39*, *EEF1A1*, *ERH*, *FTH1*, *GNAS*, *GNB2L1*, *H3F3A*, *H3F3B*, *HINT1*, *HSPA8*, *HSPCB*, *JUND*, *LDHA*, *MDH1*, *NEDD8*, *PABPC1*, *PFDN5*, *PFN1*, *PGK1*, *PRDX1*, *RAC1*, *RHOA*, *RPA2*, *RPL3|ZNF114*, *SEPT2*, *SLC25A3*, *SOD1*, *SRP14*, *SSR2*, *TEGT*, *UBB*, *UBC*, *USP11*, *VIL2*, *YWHAB*, *ZNF91* (62)	*ACTB*, *ACTG1*, *ALDOA*, *ANP32B*, *APLP2*, *ARF1*, *ARF4*, *ARPC2*, *ATF4*, *ATP5A1*, *ATP5G1*, *ATP5G3*, *ATP5O*, *ATP6V1F*, *BAT1*, *BCAP31*, *BTF3*, *CALM2*, *CANX*, *CD81*, *CFL1*, *CLN5*, *CLTA*, *COPS6*, *COX4I1*, *COX6A1*, *COX7A2*, *COX7C*, *COX8A*, *CSNK2B*, *CSTB*, *CYB5R3*, *DAZAP2*, *DDT*, *DDX39*, *DYNLL1*, *EEF1A1*, *ENO1*, *ERH*, *FTH1*, *G10*, *GAPDH*, *GNAS*, *GNB2L1*, *H3F3A*, *H3F3B*, *HINT1*, *HSPA8*, *HSPCB*, *JUND*, *KARS*, *LDHA*, *MDH1*, *MRPS12*, *MYL6*, *NACA*, *NCL*, *NEDD8*, *NONO*, *PABPC1*, *PFDN5*, *PFN1*, *PGK1*, *PHB2*, *PRDX1*, *RAC1*, *RHOA*, *RPA2*, *RPL3|ZNF114*, *RPS14*, *RPS2*, *SARS*, *SEPT2*, *SLC25A3*, *SOD1*, *SRP14*, *SSR2*, *TEGT*, *TMSB10*, *TUBB2A*, *UBB*, *UBC*, *VIL2*, *YWHAB*, *YWHAQ*, *YWHAZ*, *ZNF91* (87)	*ACTB*, *ACTG1*, *ANP32B*, *ARF1*, *ARF4*, *ARPC2*, *ATF4*, *ATP5A1*, *ATP5G1*, *ATP5G3*, *ATP5O*, *ATP6V1F*, *BAT1*, *CALM2*, *CANX*, *CLN5*, *CLTA*, *COX4I1*, *COX6A1*, *COX7A2*, *COX7C*, *COX8A*, *CSNK2B*, *CSTB*, *CYB5R3*, *DAZAP2*, *DDX39*, *EEF1A1*, *ERH*, *FTH1*, *GNAS*, *GNB2L1*, *H3F3A*, *H3F3B*, *HINT1*, *HSPA8*, *HSPCB*, *JUND*, *LDHA*, *MDH1*, *NEDD8*, *PABPC1*, *PFDN5*, *PFN1*, *PGK1*, *PRDX1*, *RAC1*, *RHOA*, *RPA2*, *RPL3|ZNF114*, *SEPT2*, *SLC25A3*, *SOD1*, *SRP14*, *SSR2*, *TEGT*, *UBB*, *UBC*, *VIL2*, *YWHAB*, *ZNF91* (61)

A4. Published papers

Kakourou G, Dhanjal S, Mamas T, Gotts S, Doshi A, Fordham K, Serhal P, Ranieri DM, Delhanty JD, Harper JC, SenGupta SB. Preimplantation genetic diagnosis for myotonic dystrophy type 1 in the UK. Neuromuscul Disord. 2008 Feb; 18(2):131-6

Dhanjal S, **Kakourou G**, Mamas T, Saleh N, Doshi A, Gotts S, Nuttall S, Fordham K, Serhal P, Delhanty J, Harper J, Sengupta S. Preimplantation genetic diagnosis for retinoblastoma predisposition. Br J Ophthalmol. 2007 Aug; 91(8):1090-1

Kakourou G, Dhanjal S, Daphnis D, Doshi A, Nuttall S, Gotts S, Serhal P, Delhanty J, Harper J, SenGupta S. Preimplantation genetic diagnosis for myotonic dystrophy type 1: detection of crossover between the gene and the linked marker APOC2. Prenat Diagn. 2007 Feb; 27(2):111-6

Souraya Jaroudi, **Georgia Kakourou**, Suzanne Cawood, Alpesh Doshi, Domenico M Ranieri, Paul Serhal, Joyce C. Harper and Sioban B. SenGupta Expression profiling of DNA repair genes in human oocytes and blastocysts using microarrays, Human Reproduction (accepted)

In preparation:

Georgia Kakourou, Souraya Jaroudi, Sarah Gotts, Alpesh Doshi, Domenico M Ranieri, Paul Serhal, Joyce C. Harper and Sioban B. SenGupta Gene expression profiling of human oocytes and embryo blastocysts (in preparation)

A5. Abstract presentations

Mamas T., **Kakourou G**., Dhanjal S., Cawood S., Doshi A., Serhal P., Xanthopoulou L., Mantzouratou A., Delhanty J., Harper J., SenGupta S. Detection of chromosomal aneuploidy in embryos from preimplantation genetic diagnosis cases for monogenic disorders Preimplantation Genetic Diagnosis International Society (PGDIS), Miami, 23-25 April, 2009.

S Dhanjal, **G Kakourou**, T Mamas, JC Harper and SB SenGupta Follow up analysis of untransferred embryos following PGD for monogenic disorders PGDIS 2009 Preimplantation Genetic Diagnosis International Society (PGDIS), Miami, 23-25 April, 2009

Seema Dhanjal, **Georgia Kakourou**, Thalia Mamas, Alpesh Doshi, Sarah Gotts, Paul Serhal, Domenico M Ranieri, Joyce C Harper, Joy DA Delhanty, Sioban B SenGupta, The pursuit of a diagnosis for every embryo in PGD British Fertility Society / ACE / Society for Reproduction and Fertility, Edinburgh 7-9 January 2009

Georgia Kakourou, Souraya Jaroudi, Suzanne Cawood, Alpesh Doshi, Paul Serhal, Joyce C Harper, Joy DA Delhanty, Sioban B SenGupta. Gene expression profiling of human oocytes and embryo blastocysts. UCL Elizabeth Garrett Anderson Institute for Women's Health, 4th annual meeting, December 2008

Kakourou G., Dhanjal S, Mamas T, Doshi A., Gotts S., Cawood S., Serhal P., Ranieri DM, Delhanty JDA, Harper JC, SenGupta SB, Inheritance of the DMPK CTG repeat in couples undergoing PGD for DM1. British Society for Human Genetics Conference, University of York, September 15-17, 2008.

Thalia Mamas, **Georgia Kakourou**, Seema Dhanjal, Alpesh Doshi, Sarah Gotts, Paul Serhal, Joy Delhanty, Joyce Harper, Sioban Sengupta. Detection of aneuploidy in embryos from PGD cases for single gene disorders. British Society for Human Genetics Conference, University of York, September 15-17, 2008

Seema Dhanjal, **Georgia Kakourou**, Thalia Mamas, Alpesh Doshi, Sarah Gotts, Paul Serhal, Domenico M Ranieri, Joyce C Harper, Joy DA Delhanty, Sioban B SenGupta, The pursuit of a diagnosis for every embryo in PGD. British Society for Human Genetics Conference, University of York, September 15-17, 2008

SenGupta S, Dhanjal S, Mamas T, Jaroudi S, **Kakourou G**, Fordhan K, Doshi A, Gotts, S, Serhal P, Harper JC, Delhanty J. Preimplantation Genetic Diagnosis for Cancer Predisposition. Prenatal Diagnosis, (2008), 28:Supplement S3, 2-3 International Society for Prenatal Diagnosis, Vancouver Canada June 1-4 2008

S Dhanjal, T Mamas, **G Kakourou**, P Serhal, J Harper, J Delhanty and S SenGupta, Preimplantation genetic diagnosis for Familial Hypercholesterolaemia Preimplantation Genetic Diagnosis International Society, Barcelona 23-26 April 2008

SenGupta S, Dhanjal S, Mamas T, Jaroudi S, **Kakourou G**, Fordham K, Doshi S, Gotts S, Serhal P, Ranieri D, Harper J and Delhanty J. Preimplantation genetic diagnosis for cancer predispostion Innovations & Progress in Healthcare for Women, Queen Elizabeth II Conference Centre, London, April 7-8, 2008

Georgia Kakourou, Seema Dhanjal, Thalia Mamas, Sarah Gotts, Alpesh Doshi, Karen Fordham, Paul Serhal, Domenico M. Ranieri, Joy DA. Delhanty, Joyce C. Harper and Sioban B. SenGupta. Preimplantation Genetic Diagnosis for Myotonic Dystrophy Type 1 in the UK. UCL Elizabeth Garrett Anderson Institute for Women's Health, 3rd annual meeting, December 2007

Kakourou G, Dhanjal S, Mamas T, Delhanty JDA, Harper JC, SenGupta SB. Investigation of the myotonic dystrophy type 1 (DM1) triplet repeat expansion and gene expression in oocytes and preimplantation embryos. UCL Elizabeth Garrett Anderson Institute for Women's Health, 3rd annual meeting, December 2007

Mamas T, **Kakourou G**, Dhanjal S, Doshi A, Gotts S, Serhal P, Delhanty J, Harper J and SenGupta SB. Detection of aneuploidy in embryos from PGD cases for single gene disorders. UCL Elizabeth Garrett Anderson Institute for Women's Health, 3rd annual meeting, December 2007

Souraya Jaroudi, Soha A. Tashkandi, Radha Bhat, Stavros Glentis, **Georgia Kakourou**, Joyce C. Harper and Sioban B. SenGupta. Assessment of MDA as a cDNA amplification technique for gene expression analysis. UCL Elizabeth Garrett Anderson Institute for Women's Health, 3rd annual meeting, December 2007

SenGupta S, Dhanjal J, Mamas T, Jaroudi S, **Kakourou G**, Fordham K, Serhal P, Harper J and Delhanty J. Difficulties of Preimplantation Genetic Diagnosis for Cancer Predisposition British Society of Human Genetics, Cancer Genetics Group, Guy's Hospital London, 7th December 2007

Kakourou G, Dhanjal S, Mamas T, Doshi A, Gotts S, Serhal P, Ranieri DM, Delhanty JDA, Harper JC, SenGupta SB. Protocol development for Preimplantation Genetic Diagnosis (PGD) of Myotonic Dystrophy Type 1 (DM1) in the UK: experience from 25 cycles., University of Milan, September 2007 6th International Myotonic Dystrophy Consortium Meeting, IDMC-6

Thalia Mamas, Seema Dhanjal, Souraya Jaroudi, **Georgia Kakourou** and Sioban SenGupta. Preimplantation Genetic Diagnosis for inherited cancer predisposition – Different Strategies. British Society for Human Genetics Conference University of York, September 2007

Georgia Kakourou, Seema Dhanjal, Thalia Mamas, Sarah Gotts, Paul Serhal, Domenico Massimo Ranieri, Joy DA. Delhanty, Joyce C. Harper and Sioban B. SenGupta. Preimplantation Genetic Diagnosis of myotonic dystrophy type 1. European Human Genetics Conference , 16-19 June 2007, Nice, France.

Seema Dhanjal, **Georgia Kakourou**, Thalia Mamas, Alpesh Doshi, Sarah Gotts, Sarah Laver, Joyce Harper, Sioban SenGupta. Does the number of cells biopsied affect the implantation of embryos in Preimplantation Genetic diagnosis? European Human Genetics Conference 16-19 June 2007, Nice, France

Georgia Kakourou, Seema Dhanjal, Richa Sud, Thalia Mamas, Alpesh Doshi, Sarah Nuttall, Sarah Gotts, Paul Serhal, Joy Delhanty, Joyce Harper, Sioban SenGupta. Preimplantation Genetic Diagnosis for Non-syndromic deafness. UCL Elizabeth Garrett Anderson Institute for Women's Health, 2nd annual meeting, December 2006

Thalia Mamas, Farah Kilani, **Georgia Kakourou**, Seema Dhanjal, Jasmin Lee, Joy Delhanty, Joyce Harper and Sioban SenGupta. Preimplantation Genetic Diagnosis for dominant monogenic disorders. UCL Elizabeth Garrett Anderson Institute for Women's Health, 2nd annual meeting, December 2006

Seema Dhanjal, **Georgia Kakourou**, Natasha Saleh, Thalia Mamas, Alpesh Doshi Sarah Gotts, Sarah Nuttal, Karen Fordham, Paul Serhal, Joy Delhanty, Joyce Harper and Sioban SenGupta. Preimplantation Genetic Diagnosis for Retinoblastoma Predisposition. UCL Elizabeth Garrett Anderson Institute for Women's Health, 2nd annual meeting, December 2006

G Kakourou, S Dhanjal, R Sud, T Mamas, A Doshi, S Nuttall, S Gotts, P Serhal, JD Delhanty, JC Harper, S SenGupta. Preimplantation Genetic Diagnosis for non-syndromic deafness. British Society for Human Genetics Conference 18-20 September, University of York, September 2006

S Dhanjal, **G Kakourou**, N Saleh, T Mamas, A Doshi, S Gotts, S Nuttall, K Fordham, P Serhal, J Delhanty, JC Harper, S SenGupta. Preimplantation Genetic Diagnosis for Retinoblastoma predisposition. British Society for Human Genetics Conference 18-20 September, University of York, September 2006

Kakourou G, Dhanjal S, Doshi A, Gotts S, Nuttall S, Serhal P, SenGupta SB. Preimplantation Genetic Diagnosis (PGD) of Myotonic Dystrophy type 1 (DM1). UCL Elizabeth Garrett Anderson Institute for Women's Health, 1st annual meeting, December 2005 (first prize for oral presentation).

Seema Dhanjal, **Georgia Kakourou**, Farah Kilani, Thalia Mamas, Nestor Phoenix, Sioban Sengupta. Preimplantation Genetic Diagnosis for inherited cancer predisposition- Strategies and problems. UCL Elizabeth Garrett Anderson Institute for Women's Health, 1st annual meeting, December 2005.